

# **AN *IN VITRO* INVESTIGATION INTO THE PIGMENTARY PHENOTYPE OF MELANOCYTE AND KERATINOCYTE CO-CULTURES TO IMPROVE WOUND HEALING**

by

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# DECLARATION

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# LIST OF ABBREVIATIONS

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|                         |   |
|-------------------------|---|
| <b>βgal</b>             | β-galactosidase   |
| <b>ddH<sub>2</sub>O</b> | double distilled water  |
| <b>μg/ml</b>            | micrograms per millilitre                                     |
| <b>bFGF</b>             | basic fibroblast growth factor                                |
| <b>BSA</b>              | bovine serum albumin  |
| <b>cm</b>               | centimetre  |
| <b>cPIC</b>             | complete proteinase inhibitor cocktail                        |
| <b>cpm</b>              | counts per minute   |
| <b>DHI</b>              | 5,6-dihydroxyindole   |
| <b>DHICA</b>            | 5,6-dihydroxyindole-5-carboxylic acid                         |
| <b>DMEM</b>             | Dulbecco's Modified Eagle medium                              |
| <b>DNA</b>              | deoxyribonucleic acid   |
| <b>DOPA</b>             | dihydroxyphenylalanine  |
| <b>ECL</b>              | enhanced chemiluminescence                                    |
| <b>EDTA</b>             | ethylenediaminetetraacetic acid                               |
| <b>ET-1</b>             | endothelin-1  |
| <b>FACS</b>             | fluorescence-activated cell sorting                           |
| <b>FCS</b>              | fetal calf serum  |
| <b>FETI</b>             | Hams F10 medium, bFGF, ET-1, TPA and IBMX (melanocyte medium) |
| <b>g/L</b>              | grams per litre   |
| <b>Greens</b>           | original keratinocyte growth medium                           |
| <b>h</b>                | hours   |
| <b>HCl</b>              | hydrochloric acid   |
| <b>IBMX</b>             | 3-isobutyl-1-methyl xanthine                                  |
| <b>ICC</b>              | immunocytochemistry   |
| <b>Kc</b>               | keratinocytes   |
| <b>kDa</b>              | kilo Dalton   |
| <b>M</b>                | molarity  |
| <b>MART-1</b>           | melanoma antigen recognised by T-cells 1                      |
| <b>Mc</b>               | melanocytes   |
| <b>mg/ml</b>            | milligram per millilitre                                      |
| <b>min</b>              | minutes   |
| <b>ml</b>               | millilitre  |
| <b>mm</b>               | millimetre  |
| <b>N</b>                | normality   |
| <b>ng/ml</b>            | nanogram per millilitre                                       |
| <b>nm</b>               | nanometre   |
| <b>nM</b>               | nanomolar   |

|                                     |   |
|-------------------------------------|---|
| <b>O<sub>2</sub>/CO<sub>2</sub></b> | oxygen/carbon dioxide                                     |
| <b>PBS</b>                          | phosphate buffered saline                                 |
| <b>qs</b>                           | quantity sufficient                                       |
| <b>RAB27A</b>                       | Ras-related protein-27A                                   |
| <b>RIPA</b>                         | radio-immunoprecipitation assay                           |
| <b>rpm</b>                          | revolutions per minute                                    |
| <b>SA-βgal</b>                      | senescence-associated beta-galactosidase                  |
| <b>SDS-PAGE</b>                     | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| <b>TBS-T</b>                        | tris-buffered saline and tween 20                         |
| <b>TE</b>                           | trypsin-EDTA  |
| <b>TPA</b>                          | 12-O-tetradecanoylphorbol-13-acetate                      |
| <b>TRP1</b>                         | tyrosinase-related protein-1                              |
| <b>TRP2</b>                         | tyrosinase-related protein-2/ dopachrome tautomerase      |
| <b>U/ml</b>                         | units per millilitre                                      |
| <b>UVR</b>                          | ultraviolet radiation                                     |
| <b>V</b>                            | voltage   |
| <b>X-gal</b>                        | 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside           |

# ABSTRACT

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## **INTRODUCTION:**

On healing, partial-thickness burn wounds usually result in depigmentation of the skin. This is due to the loss of melanocytes. The lack of pigmentation in the healed wound is particularly prominent in dark-skinned individuals and could result in serious psychosocial consequences such as low self-esteem, stigmatisation and discrimination among sufferers. Methods aimed at investigating rapid and efficient repigmentation in wounded skin are therefore pertinent. The aims of this study were two-fold:

- i) To promote melanin synthesis in human skin cells using different ratios of human melanocytes (Mc) to keratinocytes (Kc) in an *in vitro* co-culture system, in order to ensure pigmentation of the skin and,
- ii) To understand cellular mechanisms that contribute basic scientific knowledge towards clinically improved wound healing

## **METHODS:**

Ethically obtained, normal human-derived cells were co-cultured at 3 different ratios, 5Kc:1Mc (5:1), 10:1 and 20:1. Tyrosinase protein expression and activity were analysed by western blot and tyrosinase assays, respectively. Melanin assays were used to quantify the amount of melanin produced in these co-cultures. Furthermore, the morphology of the melanocytes was determined by quantification of the average number and length of dendrites per melanocyte. Scratch assays were performed to simulate a wounded skin to observe the rate of wound healing over a 4 to 48 hour period. Fluorescence microscopy was done to quantify the percentage of proliferating melanocytes in the proximity of the scratched area.

## **RESULTS:**

Western blots confirmed the presence of tyrosinase in the ratios. Interestingly, the tyrosinase expression and activity appeared to be the same between the 3 ratios. We established that the melanin assay is not a sensitive enough assay to detect the quantity

of melanin in our co-cultures. There were no morphological differences between the melanocytes cultured at the different ratios as no change was detected in dendrite number and length. In terms of wound closure between the ratios, there was no difference in the rate of migration of the epidermal cells and the percentage of proliferating melanocytes.

### **CONCLUSION:**

Overall, our results suggest that since there is no difference in the level of cellular pigmentation as well as wound healing, the 20:1 ratio of epidermal keratinocytes to melanocytes could be optimally used to enhance autologous cellular grafting. This information is considered an important contribution towards clinical application and to ensure a favourable outcome.



# CHAPTER 1: LITERATURE REVIEW

---

“I am healed but I am still different” – Sipho, 7 years old;

Red Cross War Memorial Children’s Hospital

These words echo the ongoing problem for burn victims leaving South African hospitals. Burns cases are rapidly increasing across Africa. Statistics show that it is one of the regions with the highest mortality rates as a result of fire-related injuries, with 6.1 deaths per 100000 individuals (Mock *et al.*, 2008). In South Africa, 3.2% of the population sustains burn injuries annually (Albertyn *et al.*, 2006), with recent data from Mpumalanga showing that burn fatalities are highest amongst children below 4 years of age and adults over the age of 25 (Blom *et al.*, 2011). In the Western Cape, data collected from 1997-2006 of paediatric injuries admitted to the Red Cross War Memorial Children’s Hospital in Cape Town, showed a statistic of 8.8% that was due to burn injuries, which was the 4<sup>th</sup> most common injury (Herbert *et al.*, 2012). The Tygerberg hospital burn unit recorded that 1908 patients were admitted from the period of 2003-2008 (Maritz *et al.*, 2012). This alarming trend was due to poverty, mass illiteracy, the increase in the informal settlement including the design of informal shacks, with the use of open fires as well as lifestyle factors such as alcohol usage (Albertyn *et al.*, 2006, Oluwasanmi, 1969, Van Niekerk *et al.*, 2009, Niekerk *et al.*, 2006). The most commonly affected areas on the body are the face, hands and feet (Cahill *et al.*, 2008). Often the wound treatment regimes include autologous grafting, where the patients’ own skin is used to cover the burn area. However, although a healed clinical presentation results following treatment, the wound lacks pigmentation,

reflecting a sub-optimal healing response and leading to someone such as Sipho, stating “I am healed, but I am still different”. In addition, the contrast between pigmented and non-pigmented areas is most striking in dark-skinned individuals (Kahn *et al.*, 1996). Although partial-thickness burn wounds are not life-threatening, they result in a number of psychosocial consequences, where stigmatism, low self-esteem and discrimination are part of everyday life (Dyster-Aas *et al.*, 2008, Ter Smitten *et al.*, 2011). In light of this, an understanding of the skin and its healing response at a cellular, holistic and individualistic level becomes highly pertinent. We believe that this knowledge will contribute to an optimal healing outcome.

## **1.1 The problem**

Even though treatments for partial-thickness burns are available, the remaining problem is that once the wound has healed, the outcome is often sub-optimal as the burned area remains hypopigmented and pink in colouration. This is a clinical problem, as the skin needs to be protected from ultraviolet radiation (UVR) by the pigments produced in the melanocytes (Eves *et al.*, 2005), and yet only keratinocytes are used to heal burn wounds to restore the barrier function of the skin (MacNeil, 2007). Furthermore, the discolouration gives the perception that although the wound is healed, there is still something lacking. This change in appearance results in serious psychosocial consequences such as depression (Dyster-Aas *et al.*, 2008, Ter Smitten *et al.*, 2011, Chadwick *et al.*, 2012). Moreover, burned children are often left permanently disfigured for life. The disfigurement (and consequent distress) is especially evident in the darker skinned African individuals, where the contrast between the white depigmented area

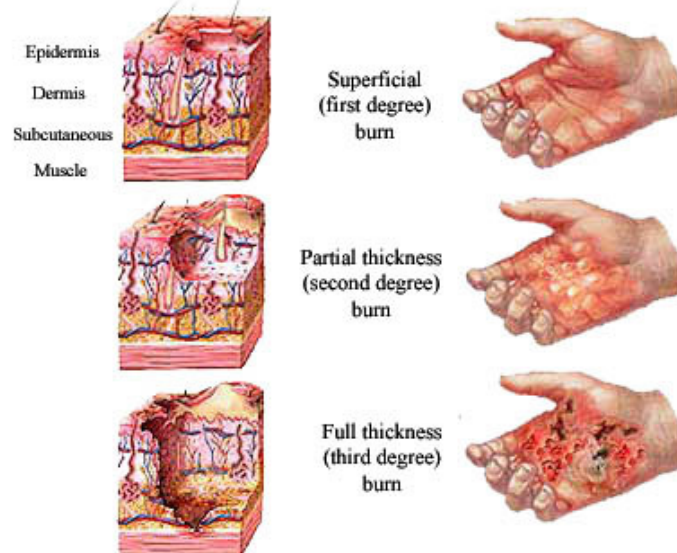
and the normally pigmented skin is particularly apparent (Kahn *et al.*, 1996). The main reason for this is that clinicians tend to concentrate, and rightly so, on the speed of healing related to keratinocyte closure, which entails the migration of keratinocytes into the wounded area by the formation of lamellipodia (Odland *et al.*, 1968). Lamellipodia are the thin cytoplasmic extensions from the front of the cell, which gives the cell a ruffled edge appearance (Small *et al.*, 2002). For repigmentation, the presence of melanocytes needs to be included in wound healing treatments. Moreover, melanocytes are needed in the environment of the keratinocytes (Imokawa *et al.*, 1998, Plonka *et al.*, 2009). Proliferation of melanocytes is under the control of keratinocytes. In order to proliferate, a model has been proposed whereby the melanocytes first retract their dendrites and detach themselves from the surrounding keratinocytes and the basement membrane, followed by proliferation and then migration along the basement membrane. The last step is to extend their dendrites and reattach themselves to the keratinocytes (Santiago-Walker *et al.*, 2009).

In a case study by Back *et al.* (2009), when the authors attempted transplantation of melanocytes and keratinocytes onto depigmented area, they noted that the repigmentation was unpredictable (Back *et al.*, 2009). In light of this, we were interested to find out if transplantation of a certain melanocyte to keratinocyte ratio could improve the level of pigmentation as well as contribute to a better healing outcome. Hence, the overall aim of this project was to promote melanin synthesis in order to repigment the skin in an *in vitro* culture system using different ratios of keratinocytes to melanocytes. These ratios included - 5 keratinocytes: 1 melanocyte (5:1), 10:1 and 20:1. This was followed by an investigation into which of the ratios

would produce a better outcome of pigmentation and at the same time, lead to improved wound healing. The comparison of 3 different ratios, to our knowledge, with regards to the pigmentary phenotype and wound healing, has never been shown before.

## 1.2 Burn wounds

Burn wounds are classified into 3 types - superficial (first degree), partial-thickness (second degree) and full-thickness (third degree) burns (Fig. 1.1). Superficial burns affect only the epidermis and are treated non-operatively and usually heal without scarring. A wound is classified as partial-thickness when the injury extends into the dermis. Full-thickness burns penetrate into the deeper portion of the dermis and causes damage to the muscle, bones and nerves (Johnson *et al.*, 2003). This project focuses on partial-thickness burns, in respect to the causes, consequences and the treatment options available.



**Figure 1.1. Classification of burn depth.** [Source: <http://www.burn-recovery.org/injuries.htm>]

Partial-thickness burns occur predominantly on the fingers, hands, wrists, feet and ankles (Kahn *et al.*, 1996). The number of burn cases in Sub-Saharan Africa is particularly alarming, especially in paediatric burns (Mock *et al.*, 2008, Albertyn *et al.*, 2006, Herbert *et al.*, 2012, Maritz *et al.*, 2012). Treatment options for partial-thickness burns include surgical and non-surgical methods. Non-surgical methods include the use of antibiotics, biological or non-biological dressings (Johnson *et al.*, 2003, Maghsoudi *et al.*, 2011, Peura *et al.*, 2012, Juhasz *et al.*, 2010, Magliacani, 1990), whereas surgical methods consist of skin grafts. Unfortunately, there is a serious lack of sufficient specialised treatment centres available for burn patients, especially in South Africa (Sowemimo, 1993). If these specialised skin grafting facilities are available, there is a deficiency of blood products and staff shortages, such as anaesthetists. The Red Cross War Memorial Children's hospital in Cape Town, consisting of 22 beds for burn cases (Cahill *et al.*, 2008), is the only unit that offers specialised treatment to children under the age of 13 (<http://childrenshospitaltrust.org.za/c2-burns-unit/>). In India, a large incidence of burns leads to a huge financial strain on an already overburdened health system. Hence, there is a need to reduce the cost of the therapy by closing the wound as early as possible without infection. This was first performed with the use of amniotic membranes. The authors found a reduction in pain and the wound healed more rapidly due to the oestrogen in the membrane (Ramakrishnan *et al.*, 1997).

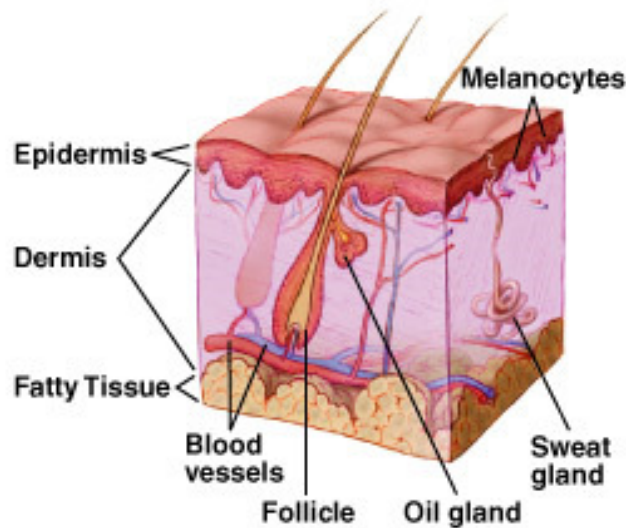
In order for us to achieve the aims set out in this project, this literature review will highlight the background knowledge that is relevant to the skin, and the cells in the epidermal layer, such as the melanocytes and keratinocytes. It will provide a brief introduction into melanin synthesis and the proteins and enzymes involved in this

process. Lastly, this literature review will shed light on the morphology of the melanocytes, specifically the dendrites, and also the mechanism of repigmentation in the skin.

### **1.3 The skin**

The skin is the largest organ of the body, with its primary function being a protective barrier – protecting the body from the external environment (Stenn *et al.*, 1992). It consists of 2 layers – the deeper layer is the dermis, overlaid by the epidermis (Fig. 1.2). The dermis, containing fibroblasts, nerves, blood vessels, adipose and connective tissue; can be further subdivided into 2 areas, where the lower part is predominantly composed of adipose tissue. The uppermost epidermal layer consists of Langerhans cells, Merkel cells, keratinocytes and melanocytes (Young *et al.*, 2000). Langerhans cells are antigen presenting cells that function as sentinels to detect foreign particles or organisms, thereby playing an important role in the immunity of the skin (Stenn *et al.*, 1992, Williams *et al.*, 1996). Merkel cells are non-neuronal cells that function as touch receptors. The keratin-producing keratinocytes make up the majority of the cell population in the epidermis; while the melanocytes provide colour to the skin through the production of melanin. The predominant cell types of the epidermis, melanocytes and keratinocytes, make up the “epidermal unit” with a ratio of 1 melanocyte to 36 keratinocytes (Fitzpatrick *et al.*, 1963). However, in the basal layer of the epidermis, the ratio becomes 1:5 (Santiago-Walker *et al.*, 2009, Fitzpatrick *et al.*, 1979). Although the skin is a 3-dimensional organ, first and second degree burn wounds predominantly

affect the epidermal and upper dermal layers. This necessitates knowledge of the interaction and function of these epidermal cellular components.

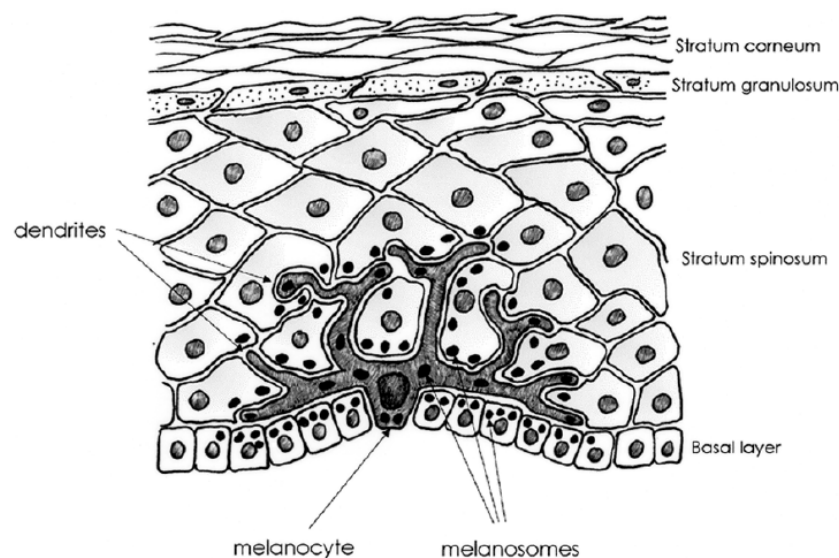


**Figure 1.2. Basic structure of the skin.** [Source: <http://fromyourdoctor.com>]

## 1.4 Epidermal cells

Melanocytes are neural crest-derived cells (Haake *et al.*, 1999). During human embryonic development, melanocyte precursor cells, melanoblasts, migrate to several target sites, including the epidermis, dermis, hair follicles, eyes, ears, heart and brain where they differentiate into mature melanocytes (Kawa *et al.*, 2000, Kawa *et al.*, 2000, Hirobe, 1992). The melanocyte precursor cells undergo differentiation, under the control of transcription factors such as SRY (sex determining region Y)-box 10, paired box 3 and microphthalmia-associated transcription factor (Hornyak *et al.*, 2001, Potterf *et al.*, 2001). In the epidermis, the melanocytes reside in the basal layer, where they interact with the surrounding keratinocytes via long thin dendritic processes (Fig. 1.3).

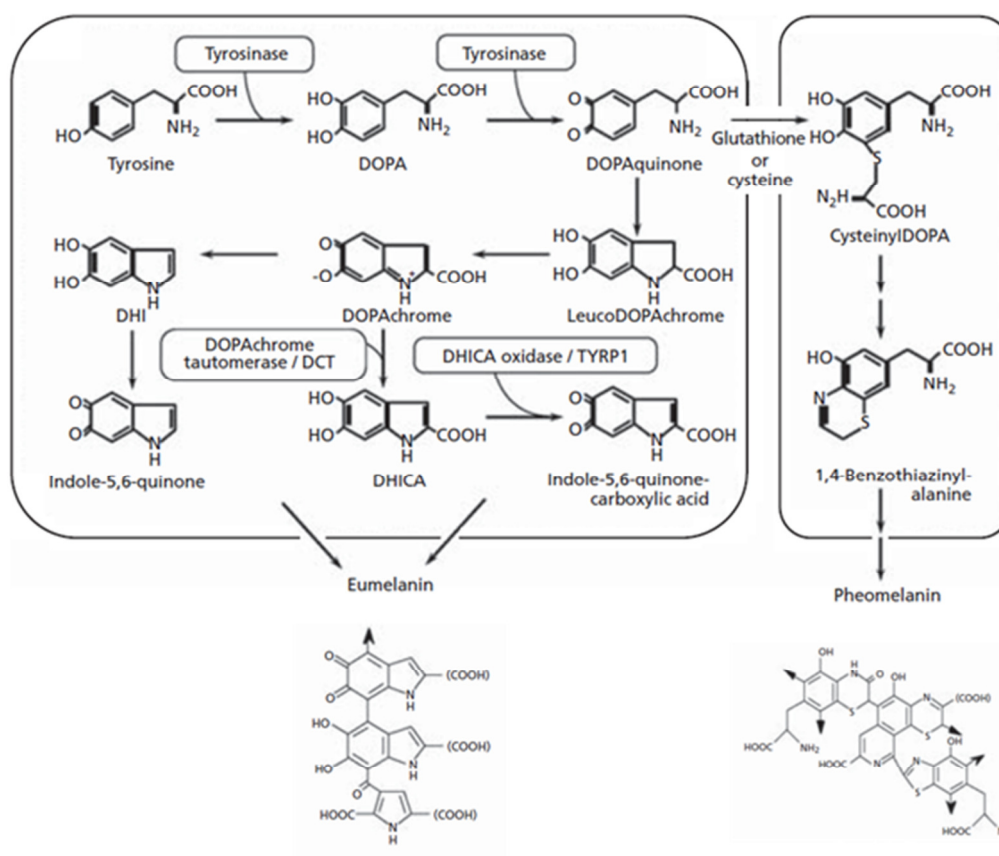
These dendrites allow for the transfer of lysosomally-derived organelles, melanosomes, to the keratinocytes (Orlow, 1995). The process of melanogenesis – formation of melanin – takes place in this organelle under the control of the enzyme, tyrosinase. Upon being taken up by the keratinocytes, they accumulate over the nucleus, effectively protecting the nuclear material against UVR and other DNA-breaking environmental factors (Eves *et al.*, 2005, Kobayashi *et al.*, 1998a). Within the melanosome, two types of melanin exist – eumelanin, the black/brown pigment; and pheomelanin, the red/brown pigment. Surprisingly, the difference in pigmentation between races is not due to the number of melanocytes, but rather depends on the amount and type of melanin produced; and the difference in size, number and distribution pattern of melanosomes within the keratinocytes (Szabo *et al.*, 1969, Tsatmali *et al.*, 2002, Alaluf *et al.*, 2003).



**Figure 1.3. The epidermis showing a melanocyte, with a number of dendrites, surrounded by the keratinocytes. [Source: Rees, 2004]**



The process of melanin formation starts with the rate limiting enzyme, tyrosinase, catalysing the hydroxylation of cytoplasmic tyrosine to dihydroxyphenylalanine (DOPA) and the subsequent oxidation of DOPA to DOPAquinone (Hearing *et al.*, 1991). At this point, the pathway splits and the 2 different types of melanin – pheomelanin and eumelanin – are produced (Fig. 1.4) (Prota, 1980). Pheomelanin, the reddish-brown pigment, is synthesised when DOPAquinone is converted to cysteinylDOPA, then 1,4-benzothiazinyl-alanine, by the addition of glutathione or cysteine. The brown-black pigment, eumelanin, is synthesised when DOPAquinone is spontaneously converted to DOPACHrome. The enzyme dopachrome tautomerase, similarly known as tyrosinase-related protein-2 (TRP2), then converts DOPACHrome to 5,6-dihydroxyindole-5-carboxylic acid (DHICA). DHICA is further oxidised by the enzyme tyrosinase-related protein-1 (TRP1) to indole-5,6-quinone-carboxylic acid. DOPACHrome can alternatively be spontaneously decarboxylated to 5,6-dihydroxyindole (DHI) (Jackson *et al.*, 1992, Tsukamoto *et al.*, 1992), which is then catalysed to indole-5,6-quinone by tyrosinase (Korner *et al.*, 1982). Indole-5,6-quinone-carboxylic acid and indole-5,6-quinone are finally polymerised to form eumelanin. Whereas, eumelanin is a photoprotective, photostable polymer; pheomelanin, in contrast, does not provide much protection against UVR and is considered a photosensitiser (Duval *et al.*, 2002). The process of melanogenesis produces many “toxic” melanin intermediates/precursors, such as phenols and quinones, which can react with and damage the macromolecules within the cells, and as such it is suggested that melanin synthesis occurs in melanosomes to protect the melanocytes (Borovansky *et al.*, 1991, Pawelek *et al.*, 1978).



**Figure 1.4. Synthesis of eu- and pheomelanin in melanogenesis.** [Modified from: Hearing, 2006 and Ito *et al.*, 2006]

Despite the mechanism of transfer, when the melanosomes appear in the cytoplasm of the keratinocytes, they form a cap over the nucleus to protect the DNA from damage caused by UVR (Kobayashi *et al.*, 1998a). Interestingly, in an *in vitro* study, Minwalla *et al.* (2001) demonstrated that the distribution of melanosomes is dependent on the ethnic/racial origin of the keratinocytes, rather than the size of the melanosome. They showed that in keratinocytes from light-skinned individuals, 4 to 8 melanosomes were distributed in clusters in the perinuclear area. However, melanosomes in keratinocytes from dark-skinned individuals were distributed as singular melanosomes (Minwalla *et al.*, 2001, Thong *et al.*, 2003, Yoshida *et al.*, 2007). Moreover, the size of these

individual melanosomes is usually larger, approximately 800 nm in length, compared to those found in light-skinned keratinocytes, approximately 400 nm (Szabo *et al.*, 1969, Minwalla *et al.*, 2001, Kollias *et al.*, 1991, Konrad *et al.*, 1973). Degradation of melanosomes in the keratinocytes is not clearly understood; however in an *in vitro* system using melanosomes isolated from SKMEL-188 melanoma cells or normal human melanocytes, it was shown that melanosomes were more rapidly degraded in keratinocytes from light-skinned individuals, compared to those from dark skin (Ebanks *et al.*, 2011). This could suggest that darker-skinned individuals may have a different healing response compared to light-skinned individuals. However, to our knowledge no literature corroborates this suggestion.

#### *1.4.1 Proteins and enzymes involved in melanogenesis*

As the process of melanogenesis is critical to restoring pigmentation to healing skin, knowledge of the proteins involved in this process is essential. Moreover, immunocytochemical analyses of healed skin use these proteins as markers of differentiated melanocytes. These crucial enzymes involved in melanogenesis are tyrosinase, TRP1 and TRP2. The rate-limiting enzyme of the melanogenesis pathway, tyrosinase, is a 60 kDa protein that undergoes post-translational modification by glycosylation to become a mature enzyme in the endoplasmic reticulum and Golgi apparatus. After glycosylation, the molecular weight of tyrosinase is approximately 70-80 kDa, depending on the number of sites that have been glycosylated (Ujvari *et al.*, 2001, Halaban *et al.*, 1997). Tyrosinase has 6 N-linked glycosylation sites and mutation at any of these sites decreases its function. The mature enzyme is transported into

melanosomes via clathrin-coated vesicles (Watabe *et al.*, 2004), where tyrosinase initiates melanogenesis. Tyrosinase is structurally similar to TRP1 and TRP2, and highly conserved between different species (Hearing *et al.*, 1991). Tyrosinase has copper ions in its metal binding regions, whereas TRP2 has zinc. The metal co-factor for TRP1 is currently unknown. The molecular weight of TRP1, depending on the level of glycosylation, and TRP2 are 70-90 kDa and 75 kDa, respectively. All 3 enzymes are localised on melanosomal membranes. TRP1 is needed for the correct trafficking of tyrosinase into the melanosomes (Toyofuku *et al.*, 2001, Manga *et al.*, 2000) as well as stabilising tyrosinase activity (Kobayashi *et al.*, 1998b). TRP2 is involved in tyrosinase stabilisation within the melanosome (Manga *et al.*, 2000).

Other proteins involved in melanogenesis include Pmel17, melanoma antigen recognised by T-cells 1 (MART-1 or Melan-A) and p protein. Pmel17, also known as gp100 or SILV, is a structural protein that forms the fibrils in stage I and II melanosomes (pre-melanosomes) (Kushimoto *et al.*, 2001, Berson *et al.*, 2001, Yasumoto *et al.*, 2004). MART-1, a hydrophobic transmembrane protein also found in the pre-melanosomes, is necessary for melanosome maturation as it regulates the expression, stability, trafficking and processing of Pmel17 (Hoashi *et al.*, 2005). P protein, found in the melanosome, is needed for the regulation of intramelanosomal pH (Puri *et al.*, 2000).

### 1.4.2 Dendricity/Morphology

Dendrites, the cytoplasmic extensions of melanocytes, are an important morphological feature that allows the interaction between the cells of the epidermal melanin unit. It is also necessary for the transfer of melanosomes from the melanocytes to the keratinocytes (Tsatmali *et al.*, 2002, Jeon *et al.*, 2007, Costin *et al.*, 2007). Melanogenesis is associated with dendricity in that an increase in the process of melanogenesis correlates with an increase in the number and length of the dendrites (Kim *et al.*, 2010). It follows therefore that length and number of dendrites reflects the differentiated status of the melanocyte. Melanocyte mono-cultures usually display characteristic dendrites with short bipolar phenotype. However, in melanocyte and keratinocyte co-cultures, the dendrites are typically long and poly-dendritic, extending to be in contact with the keratinocytes. Melanocytes that are too far from keratinocytes display shorter dendrites compared to those that are in direct contact with the keratinocytes (Nakazawa *et al.*, 1995). In studies performed with keratinocyte-conditioned medium, it was shown that following ultraviolet radiation, keratinocyte-derived factors were increased which led to increased growth, melanisation and dendricity. Keratinocytes produce and secrete many factors, such as  $\alpha$ -melanocyte-stimulating hormone, that are involved in the regulation of melanocyte proliferation, pigmentation and influence dendricity (Hunt *et al.*, 1996). Other keratinocyte-derived factors include fibroblast growth factor (proliferation), nerve growth factor (Yaar *et al.*, 1994), prostaglandin E<sub>2</sub> (dendricity; (Scott *et al.*, 2007) and endothelin-1 (proliferation, pigmentation and dendricity; (Hara *et al.*, 1995, Imokawa, 2004, Imokawa *et al.*, 1992,

Halaban *et al.*, 1988). These factors are therefore very important in the interaction between melanocytes and keratinocytes.

## **1.5 Mechanisms of repigmentation**

Melanocyte precursors have been shown definitively to be located in the hair follicle bulge which acts as a niche (Nishimura, 2011, Tanimura *et al.*, 2011). For the migration of melanocytes into the depigmented areas following wounding, the architecture and adhesion mechanisms of the cell and its surrounding matrix need to be altered. The extracellular matrix is remodeled to allow the migration of these melanocytes. This requires a balance between the synthesis and degradation of collagen, proteoglycan and multi-adhesive matrix proteins by matrix metalloproteases and their inhibitors (Kumar *et al.*, 2011). Thus, repigmentation occurs due to stimulation and migration of melanocyte precursors into the unpigmented regions where they differentiate and produce pigment. In the case of burns, the hair follicle is often destroyed. In light of the current dogma, this would then lead to a lack of melanocyte precursor cells and an associated absence of pigment. However, our finding that glabrous (hair-less), vitiliginous skin could repigment after treatment, suggested that melanocyte precursor niches possibly also exist outside the hair follicle (Davids *et al.*, 2009). Despite this, healed skin remains with a lack of pigmentation, necessitating extra melanocytes to be grafted onto the depigmented regions. Options to repigment the skin are available but involve surgery. This includes split-thickness skin grafting (Taki *et al.*, 1985), skin chip grafting (Harashina *et al.*, 1985), punch grafting (Orentreich *et al.*, 1972, Fujii *et al.*, 2007), sheet grafting and cultured epithelial autografts (Falabella, 1971, Suvanprakorn

*et al.*, 1985). Punch grafting is performed with the use of a biopsy punch to remove small pieces of skin from the donor site, while sheet grafts are larger pieces of skin obtained from the donor site with a dermatome. Split-thickness skin grafts using the patient's own skin from a donor site is the gold standard of wound healing (Juhasz *et al.*, 2010). However, an advantage of using either cultured or non-cultured autologous cellular grafts is that a small donor area can be used to cover a larger recipient area.

Despite the burden of burns remaining a significant problem in South Africa, we believe that the approach taken by this project will make an important contribution towards clinical application, with respect to increasing the efficacy of autologous cellular grafting, and lead to a better healing outcome.

## CHAPTER 2: MATERIALS AND METHODS

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### 2.1 Tissue culture

#### 2.1.1 Primary cells and cell line used

Primary melanocytes were aseptically isolated from human newborn (<1 year) foreskin using a modified method described by Eves *et al.* (2005). Briefly, the samples were transported in tubes containing Dulbecco's Modified Eagle's medium (DMEM; Appendix A.5) with antibiotics (100 U/ml penicillin/100 µg/ml streptomycin; Appendix A.1), then cut into 3-5 mm thin strips and placed in 5 mg/ml dispase II (Sigma-Aldrich, USA) in Hanks buffered salt solution (Appendix A.2) at 4°C overnight. The following day, the epidermal sheet was removed with forceps and placed into a drop of phosphate buffered saline (PBS, Appendix A.3) and manually disaggregated into smaller pieces with a scalpel (KIMIX, RSA) before being transferred into a 0.25% trypsin, 0.05% EDTA solution (pH 7.5, Appendix A.4) at 37°C. The epidermal sheets were triturated every 5 min and after 15 min, 10% Fetal Calf Serum (FCS; GIBCO, USA) was added to inactivate the trypsin. The cell suspension was then filtered through a 70 µM cell strainer (BD Biosciences, USA) to remove larger pieces of tissue that had not dissociated into a cell suspension. After centrifugation at 2000 rpm for 5 min, the cell pellet was resuspended in melanocyte growth medium, FETI (Table 2.1) and incubated at 37°C with 5% CO<sub>2</sub>/95% O<sub>2</sub> (O<sub>2</sub>/CO<sub>2</sub> incubator; Sanyo, Japan) overnight. No melanocyte cultures used in this project exceeded passage 9.



**Table 2.1. List of components in the melanocyte growth medium, FETI**

| <b><u>MEDIUM</u></b> | <b><u>COMPONENT</u></b>              | <b><u>CONCENTRATION</u></b>              | <b><u>SOURCE</u></b>     |
|----------------------|--------------------------------------|--|--------------------------|
| FETI                 | Hams F10                             | 9.86 g/L with 1.2 g/L sodium bicarbonate | Highveld Biological, RSA |
|                      | Fetal Calf Serum                     | 2%                                       | GIBCO, USA               |
|                      | Ultrosor G                           | 1%                                       | PALL Life Sciences, USA  |
|                      | Human Fibroblast Growth Factor 2     | 2 ng/ml                                  | Miltenyi Biotec, Germany |
|                      | Endothelin 1                         | 2 ng/ml                                  | Sigma-Aldrich, USA       |
|                      | 12-O-tetradecanoylphorbol-13-acetate | 16 nM                                    | Sigma-Aldrich, USA       |
|                      | 3-isobutyl-1-methyl xanthine         | 0.05 mM                                  | Sigma-Aldrich, USA       |
|                      | Penicillin/streptomycin              | 100 U/ml; 100 µg/ml                      | Sigma-Aldrich, USA       |

The spontaneously immortalised human keratinocyte cell line, HaCaT, a gift from Prof Fusenig, Germany, was used in all co-cultures with primary human melanocytes (Boukamp *et al.*, 1988). Melanocytes and HaCaTs were prepared and grown in their own specialised medium. Melanocytes were cultured in FETI medium modified from Smit *et al.* (2008), while HaCaTs were cultured in DMEM. The co-culture medium, Greens, modified from Rheinwald and Green (1977) was used when the melanocytes and HaCaTs were cultured together for the different assays (Table 2.2) (Rheinwald *et*

*al.*, 1977). Cells were grown in a humidified incubator (Sanyo, Japan) with 5% CO<sub>2</sub>/95% O<sub>2</sub> at 37°C.

**Table 2.2. List of components in co-culture medium, Greens**

| <b><u>MEDIUM</u></b> | <b><u>COMPONENT</u></b>                  | <b><u>CONCENTRATION</u></b> | <b><u>SOURCE</u></b>                         |
|----------------------|--|-----------------------------|--|
| Greens               | DMEM/F12                                 | 1:1                         | GIBCO, USA                                   |
|                      | Insulin                                  | 5 µg/ml                     | NovoRapid,<br>Novo Nordisk<br>(Pty) Ltd, RSA |
|                      | Hydrocortisone                           | 0.4 µg/ml                   | Sigma-Aldrich,<br>USA                        |
|                      | Cholera toxin                            | 0.1 nM                      | Sigma-Aldrich,<br>USA                        |
|                      | 3, 3', 5-Triiodo-L-thyronine sodium salt | 2 nM                        | Sigma-Aldrich,<br>USA                        |
|                      | Transferrin                              | 5 mg/ml                     | Sigma-Aldrich,<br>USA                        |
|                      | Epidermal growth factor                  | 10 ng/ml                    | Sigma-Aldrich,<br>USA                        |
|                      | Penicillin/streptomycin                  | 100 U/ml; 100µg/ml          | Sigma-Aldrich,<br>USA                        |
|                      | Gentamycin                               | 0.2 mg/ml                   | Sigma-Aldrich,<br>USA                        |
|                      | Sodium bicarbonate                       | 0.075%                      | Merck, RSA                                   |
|                      | HEPES buffer                             | 20 mM                       | Sigma-Aldrich,<br>USA                        |
|                      | Fetal Calf Serum                         | 10%                         | GIBCO, USA                                   |

### *2.1.2 Trypsinisation*

In order to propagate cells in culture, they are passaged when the tissue culture dishes are 80~100 % confluent. This was performed by incubating the primary melanocytes with a 0.05 % trypsin/ 0.02% EDTA solution (TE, Appendix A.28) for 3~5 min, followed by the addition of FETI to inactivate the trypsin activity. The cells were triturated and centrifuged at 2500 rpm for 3 min. The cell pellet was then resuspended in FETI and placed in new sterile tissue culture dishes. In the case of passaging the HaCaTs, cells were initially incubated with 0.05 % EDTA (Appendix A.27) for 15 min, followed by an incubation with TE for 3~5 min. The cells were triturated following the addition of DMEM, and then centrifuged. The cell pellet was resuspended in DMEM and cells were transferred to new dishes. Trypsinisation of the co-cultures was performed similarly to the HaCaTs, however, Greens was used to inactivate the trypsin and resuspend the cells.

## **2.2 Experimental setup**

Three ratios of co-cultures of keratinocytes to melanocytes were used. These included 5:1, 10:1 and 20:1 keratinocytes to melanocytes, respectively.

In the following tables, the numbers of cells seeded for each experiment is shown. The number of melanocytes used was kept constant in the different assays shown in Tables 2.3 and 2.4. In the scratch assays (Table 2.5) the melanocyte cell numbers were varied

as the same cell confluency was needed before the scratch (Chapter 2.10) was to be performed.

**Table 2.3. Number of cells used for senescence-associated beta-galactosidase and dendricity assays**

| <b><u>Ratio</u></b> | <b><u>Keratinocytes</u></b> | <b><u>Melanocytes</u></b> |
|---------------------|-----------------------------|---------------------------|
| 5:1                 | $5 \times 10^4$             | $1 \times 10^4$           |
| 10:1                | $1 \times 10^5$             | $1 \times 10^4$           |
| 20:1                | $2 \times 10^5$             | $1 \times 10^4$           |

**Table 2.4. Number of cells used for western blot, melanin and tyrosinase enzymatic activity assays**

| <b><u>Ratio</u></b>  | <b><u>Keratinocytes</u></b> | <b><u>Melanocytes</u></b> |
|----------------------|-----------------------------|---------------------------|
| 5:1                  | $1 \times 10^5$             | $2 \times 10^4$           |
| 10:1                 | $2 \times 10^5$             | $2 \times 10^4$           |
| 20:1                 | $4 \times 10^5$             | $2 \times 10^4$           |
| Keratinocyte control | $2 \times 10^5$             | -                         |
| Melanocyte control   | -                           | $5 \times 10^5$           |

**Table 2.5. Number of cells used for scratch assays**

| <b><u>Ratio</u></b>  | <b><u>Keratinocytes</u></b> | <b><u>Melanocytes</u></b> |
|----------------------|-----------------------------|---------------------------|
| 5:1                  | $1.5 \times 10^5$           | $3 \times 10^4$           |
| 10:1                 | $1.5 \times 10^5$           | $1.5 \times 10^4$         |
| 20:1                 | $1.5 \times 10^5$           | $0.75 \times 10^4$        |
| Keratinocyte control | $1.5 \times 10^5$           | -                         |
| Melanocyte control   | -                           | $1 \times 10^5$           |

### **2.3 Senescence-associated beta-galactosidase (SA-βgal) assay**

The SA-βgal assay detects cells undergoing senescence and determines whether certain morphological aspects can provide an indication of senescent cells (Debacq-Chainiaux *et al.*, 2009). Although all cells express β-galactosidase activity, detected at pH 4.0, only senescent cells express β-galactosidase activity at pH 6.0. This enzymatic activity can be observed when the 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal) substrate is cleaved by β-galactosidase to form an insoluble blue compound (Dimri *et al.*, 1995). In our experimental system, melanocytes, in mono-cultures and co-culture with HaCaTs, were seeded onto 35 mm<sup>2</sup> glass coverslips, allowed to adhere for 48 h, washed with 1x PBS (pH 6), and fixed with 4% paraformaldehyde (Merck, Germany; Appendix A.6) at room temperature for 15 min. Following further PBS washes, the βgal stain (1 mg/ml; Appendix A.7), was added to the cells and incubated at 37°C for 12-16 h. The cells were then washed and mounted onto glass slides with mounting medium, mowiol (Appendix A.8), at room temperature overnight. The slides were viewed under brightfield at 400 x magnification using the Zeiss Axioskop 200 (Carl Zeiss Microscopy, Germany) microscope. Images were captured on AxioCam HR (Carl Zeiss Microscopy, Germany) with the associated AxioVision 4.7 software (Carl Zeiss Microscopy, Germany). Senescent cells were recognised by a bluish tinge.

### **2.4 Western blot analyses: Tyrosinase protein expression**

Western blot analysis is an indirect method used to detect proteins of interest by resolving the sample protein on polyacrylamide gels, followed by the electrophoretic

transfer of the protein onto membranes. This is followed by detection using specific antibodies to the protein(s) of interest followed by enhanced chemiluminescence (ECL) detection on an x-ray film (Towbin *et al.*, 1979, Alegria-Schaffer *et al.*, 2009). This study employed western blots to confirm the presence of tyrosinase protein expression in the co-cultures. Briefly, cells were cultured as described in Table 2.4. After the experimental period (6 days), proteins were extracted on ice by adding complete radio-immunoprecipitation assay (RIPA) buffer (Appendix A.10) to the dish of cells. A cell scraper was then used to scrape all the cells to the bottom of the angled dish. The lysed cells were subsequently transferred to a 1.5 ml microfuge tube, vortexed and placed at -80°C for 60 min to aid in the removal of contaminating DNA. Once thawed, on ice, the samples were centrifuged at 12000 rpm at 4°C for 20 min. The supernatant was transferred to a clean 1.5 ml microfuge tube and stored at -80°C till further analyses were conducted.

The protein concentration of the samples was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, USA). This kit is a colorimetric assay whereby a purple-coloured reaction product is produced when two bicinchoninic acid molecules chelate with one cuprous ion. This product is then read spectrophotometrically (Versamax microplate reader, Molecular Devices, USA) at 562 nm and compared to a standard curve generated using bovine serum albumin (Sigma-Aldrich, USA) at known concentrations. The protein concentration of each sample is then calculated using SoftMax Pro software (Version 4.3.1 Life Sciences Edition, USA).

Eighty micrograms of protein with an associated quantity of loading dye (Appendix A.16) was denatured on the heating block (AccuBlock Digital Dry Bath; Labnet International, USA) at 85°C for 5 min. Once cooled to room temperature, the protein samples, together with the molecular weight peqGOLD protein marker IV (PEQLAB Biotechnologie, Germany), were resolved on a 10% SDS-PAGE gel (Appendix A.18) with an initial stacking gel (4%, Appendix A.21) at 100 V in running buffer (Appendix A.22) and transferred onto a nitrocellulose membrane (Amersham Hybond ECL; GE Healthcare, UK) at 100 V in transfer buffer (Appendix A.23) at room temperature for 60 min using the Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories, USA). The membrane was then rinsed with tris-buffered saline-tween 20 (TBS-T, Appendix A.25) solution, blocked with 5% fat-free milk in TBS-T (Appendix A.26) at room temperature for 60 min. Tyrosinase was detected by incubating the membrane with an anti-tyrosinase primary antibody, Table 2.6, at 4°C overnight. The following day, the membrane was washed with TBS-T, and incubated with the secondary antibody, donkey anti-goat conjugated to horseradish peroxidase (Table 2.6), at room temperature for 1 h on a shaker. This was followed by another wash with TBS-T and WesternBright ECL (Advansta, USA) was added according to manufacturer's instructions. The membrane was then exposed to medical x-ray film (AGFA, Belgium) in the dark, developed (AGFA, Belgium) and fixed (AGFA, Belgium) before rinsing with water and air-dried. To re-detect another protein, the membrane was "stripped" by several washes in TBS-T. The membrane was then incubated with the primary antibody, p38 (Table 2.6), to determine the loading control for the proteins. The unphosphorylated p38 is used as a control because it is highly expressed and remains constant in cells. The same procedures were followed as previously described with the tyrosinase. The x-ray film

was scanned with G:BOX ChemiXT-16 (SynGene, England) using the GeneSnap software (version 7.07, SynGene, England) and analysed for densitometry with GeneTools (version 4.00, SynGene, England).

**Table 2.6. Concentration of primary and secondary antibodies used**

| <b>Primary antibody</b>   | <b>Concentration</b>       | <b>Secondary antibody</b>  | <b>Concentration</b>       |
|---|----------------------------|--|----------------------------|
| MART-1 (Cat: Melan-A: sc-20032, Santa Cruz Biotechnology, USA)              | 1:200 in 10% BSA           | Donkey anti-mouse Alexa-488 (Cat: NL009, R&D Systems, USA)                                       | 1:500 in 10% BSA           |
| Ki-67 (Cat: ab15580, rabbit polyclonal, Abcam, UK)                          | 1:500 in 10% BSA           | Donkey anti-rabbit Cy-3 (Cat: 711-166-152, AEC-Amersham, RSA)                                    | 1:1500 in 10% BSA          |
| Tyrosinase (C-19; Cat: sc-7833, Santa Cruz Biotechnology, USA)              | 1:1000 in 5% fat-free milk | Donkey anti-goat Horseradish peroxidase conjugated (Cat: sc-2020, Santa Cruz Biotechnology, USA) | 1:5000 in 5% fat-free milk |
| p38 (Cat: p-38 MAP Kinase: MO800 rabbit polyclonal, Sigma-Aldrich, Germany) | 1:5000 in TBS-T            | Goat anti-rabbit Horseradish peroxidase conjugate (Cat: 170-6515, Bio-Rad Laboratories, USA)     | 1:5000 in 5% fat-free milk |

## 2.5 Tyrosinase enzyme activity assay

In melanogenesis, tyrosine is hydroxylated to dihydroxyphenylalanine (DOPA) and then oxidised to DOPAquinone. These initial 2 steps are catalysed by the same enzyme, tyrosinase. Since tyrosinase is the rate-limiting enzyme in this pathway, this assay is designed to determine the level of enzyme activity with the addition of DOPA and radiolabelled  $^{14}\text{C}$  tyrosine to drive the reaction. The cellular proteins isolated from melanocytes or co-cultures with melanocytes contain tyrosinase, which then hydroxylates the  $^{14}\text{C}$  tyrosine to DOPA, and oxidises the radiolabelled DOPA to DOPAquinone. The radioactive tyrosine is incorporated into DOPA and DOPAquinone which are then measured in this assay. DOPA and DOPAquinone are products of



tyrosinase enzyme activity, therefore the amount of incorporated radioactive tyrosine into these products are measured as an indication of tyrosinase activity. The incorporation of  $^{14}\text{C}$  tyrosine allows one to detect the melanin and its radiolabelled intermediates (Hearing *et al.*, 1976, Oikawa *et al.*, 1972). This assay was modified from the original protocol described by Hearing *et al.*, 1976. Briefly, cells were cultured in 10 cm diameter tissue culture dishes for 6 days in their respective ratios (Table 2.4). On day 6, cells were lifted with 0.05% EDTA (Appendix A.27), followed by trypsinisation with TE (Appendix A.28). Following centrifugation, the cell pellet was washed with PBS and transferred to a 1.5 ml microfuge tube on ice. The PBS wash procedure was repeated to remove all traces of medium, following which the pellet was re-suspended by vortexing in 0.1 M sodium phosphate buffer (pH 7.2, Appendix A.29) containing 0.5% Triton X-100 (Appendix A.14) and 1 x cPIC (Roche Diagnostics, Germany; Appendix A.11) for approximately 1 min. The samples were repeatedly freeze-thawed in liquid nitrogen and then centrifuged at 12000 rpm at 4°C for 20 min. The supernatant was transferred to clean 1.5 ml microfuge tubes and stored at -80°C till needed.

Once sample protein concentration was determined, 150 µg/ml was incubated in a  $^{14}\text{C}$  tyrosine (20 µCi/ml; American Radiolabeled Chemicals, USA) and 0.25 mM DOPA (Sigma-Aldrich, USA; Appendix A.31) solution at 37°C for 1 h. Samples were then transferred to glass microfiber filters with a diameter of 25 mm (Whatman, England) and left to air-dry. The microfiber filters have a specific size, therefore the radioactive tyrosine incorporated DOPA and DOPAquinone will bind to the filters. Once dry, the discs were washed 3 times in 0.1 N HCl (Appendix A.32) at room temperature for 15 min per wash, followed by 2 further 5 min washes in 95% ethanol (Merck, RSA) and a

final 5 min wash in acetone (Merck, RSA). These wash steps were performed to remove the radioactive tyrosine on its own, that were too small to bind to the filter discs. The discs were then placed in scintillation vials (Sigma-Aldrich, USA) and left to air-dry overnight. The following day, 5 ml scintillation fluid (Quicksafe A; Zinsser Analytic, Germany) was added to each vial and the samples were read on a Tri-Carb 2100 TR liquid scintillation analyser (Packard, USA). Results were expressed as counts per minute (cpm).

## 2.6 Melanin assay

The culmination of melanogenesis results in the production of a mixture of melanin (Fig. 1.4). The melanin assay quantifies the total amount of melanin in the co-cultures. This assay was performed as previously described (Oka *et al.*, 1996). Briefly, cells were cultured as previously described (Table 2.4) for a period of 6 days. One million cells ( $1 \times 10^6$ ) were centrifuged, the supernatant removed and the pellet washed with 1 x PBS. This process was repeated and the cell pellet was re-suspended in 1 ml NaOH (1 M; Appendix A.33) and heated to 100°C for 30 min on a heating block (AccuBlock Digital Dry Bath; Labnet International, USA) to destroy the cell membrane and solubilise the melanin to be measured. Samples were cooled to room temperature for 30 min, before being centrifuged at 16000 x *g* for 20 min. The supernatant was read at 400 nm on a spectrophotometer (Versamax microplate reader; Molecular Devices, USA) in triplicate. Results were compared to a standard curve prepared from synthetic melanin (prepared by oxidation of tyrosine with hydrogen peroxide; Sigma-Aldrich, USA). Results are presented as µg/ml.

## **2.7 Fluorescence-activated cell sorting (FACS)**

In order to analyse melanocytes for the melanin assay following a period of co-culture, melanocytes and keratinocytes were separated by cell size and granularity by means of a flow cytometer. However, before attempting to separate the co-cultures for analysis, mono-cultures of melanocytes and keratinocytes were trypsinised as previously described, centrifuged and the cell pellets resuspended in PBS. The cells were then counted and  $1 \times 10^6$  cells were transferred into 5 ml BD Falcon round-bottom tubes (BD Biosciences, USA), in 500  $\mu$ l PBS. A “mixed” 1:1 ratio of keratinocytes to melanocytes was transferred into another tube. Cells were then sent through the FACSCalibur (BD Biosciences, USA) and analysed with CellQuest Pro (v5.2.1; BD Biosciences, USA).

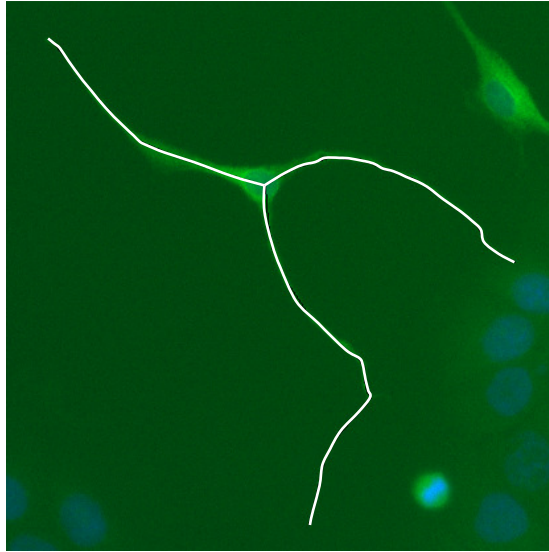
## **2.8 Immunocytochemistry**

Immunocytochemistry (ICC) is a method whereby proteins of interest can be visualised using antibodies targeted to a specific protein or antigen (Van Noorden *et al.*, 1983). It is utilised in morphological analyses and in the detection of the location of the proteins or antigens, on cells adhered to glass coverslips or on sections of tissue. Microscopes are used to visualise the antibodies bound to the proteins of interest. The methodology described below utilises the indirect method to detect specific proteins/antigens. A primary antibody is added to the cells which will target the antigen, and then a secondary antibody, conjugated to a fluorophore, is added and will bind to the primary antibody. A fluorescent microscope is used to excite/stimulate the fluorophore and the emission is visualised. In this project, ICC was used to detect epitopes expressed during

both the dendricity (Chapter 2.9) and scratch (Chapter 2.10) assays. This was to enhance the visualisation of the melanocytes within the co-cultures. In the case of the scratch assays, ICC was performed to determine whether melanocytes were proliferating as they migrated into the denuded area following the scratch. The protocol used to stain the cells was modified from Cunningham (1994). Briefly, cells adhered to the 35 mm<sup>2</sup> glass coverslips (from dendrite quantification and scratch assays) were fixed with 4% paraformaldehyde at room temperature for 15 min, then washed with 1 x PBS and permeabilised with 0.1% Triton X-100 (Appendix A.34) for 15 min, with a change of Triton X-100 after the initial 5 min. Following another PBS wash, the cells were then incubated with the primary antibodies (Table 2.6) diluted in 10% bovine serum albumin (BSA, Appendix A.35) - either MART-1 only, in the case of the dendrite quantification assay; or MART-1 with Ki-67 for the scratch assays. MART-1 is specific for the melanocytes as it binds to the transmembrane protein on the Stage I and II pre-melanosomes (Coulie *et al.*, 1994, Kawakami *et al.*, 1994, Delevoye *et al.*, 2011). Ki-67 is a nuclear marker for proliferation (Scholzen *et al.*, 2000). It is expressed in proliferating cells in the different stages of the cell cycle, except for cells in the resting G<sub>0</sub> phase. After incubating on ice at 4°C for 30 min, unbound antibodies were washed off, followed by incubation with the secondary antibodies (Table 2.6) diluted in 10% BSA at 4°C for a further 30 min. Another wash was performed before the coverslips were mounted onto glass slides with mowiol containing n-propyl gallate (Appendix A.36; Sigma-Aldrich, USA).

## 2.9 Dendrite quantification assay

One melanocyte can be in contact with approximately 36 keratinocytes (Fitzpatrick *et al.*, 1963). This is due to their extensive dendrite unit. These cellular processes are the conduits for melanosome transfer from the melanocytes to keratinocytes. The transferred melanosomes would then provide keratinocytes protection against UVR. It has previously been shown that the dendrites increase in number and length following UVR. This is to accommodate for the increase in melanosome transfer (Friedmann *et al.*, 1987). Thus, the quantification of dendrites can be used as an indicator for increased melanogenesis and hence, melanocyte differentiation (Kim *et al.*, 2010). For this assay, cells were seeded onto 35 mm diameter (35 mm) tissue culture dishes for the 5:1 ratio, while 6 cm diameter (6 cm) dishes were used for both 10:1 and 20:1 ratios. After 4 days of culture, cells were then fixed in 4% paraformaldehyde and stained with the anti-MART1 antibody (1 in 200 dilution, Table 2.6) to visualise the melanocytes. Immunofluorescent images were taken on an inverted fluorescent microscope (AxioVert 200M; Carl Zeiss Microscopy, Germany) with the AxioCam HRm camera (Carl Zeiss Microscopy, Germany) and AxioVision version 4.8 software (Carl Zeiss Microscopy, Germany) at 100 x magnification. The number of dendrites per melanocyte was counted and the length of each dendrite measured using Image J software (version 1.43u; National Institutes of Health, USA). Melanocytes were counted in 10 random fields of view and dendrites measured starting from a central point in the nucleus radiating outwards to the tip of each dendrite (black lines draw onto a melanocyte in Fig. 2.1).



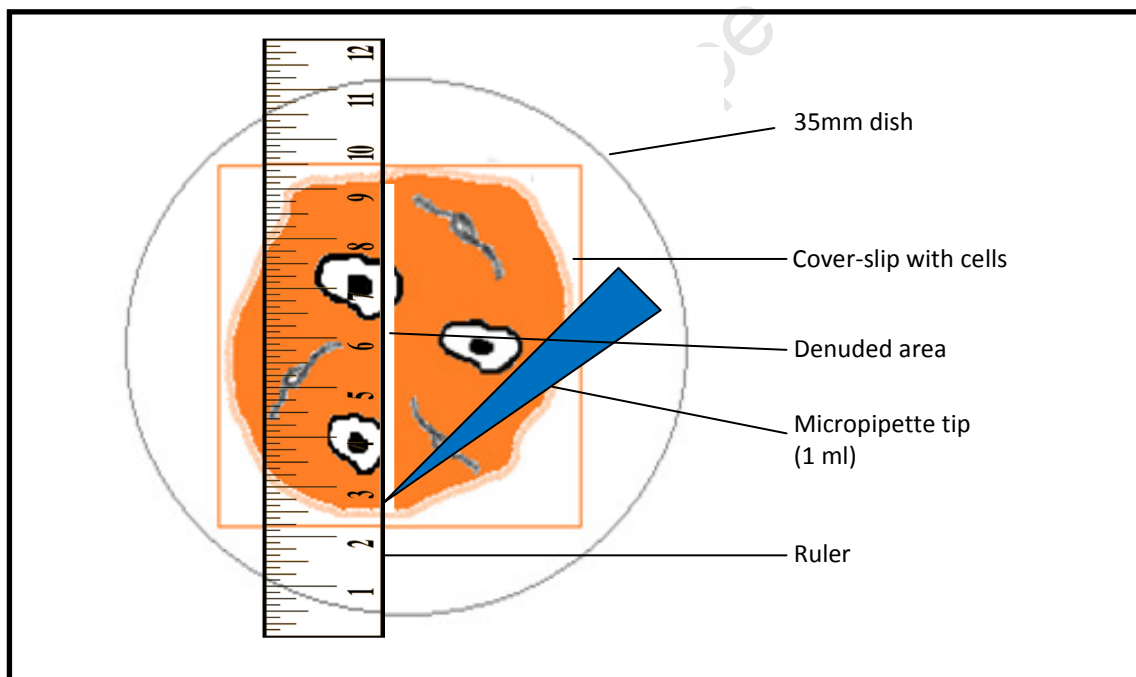
**Figure 2.1.** Melanocytes stained with anti-MART-1 antibody (green) and nuclei stained with Hoechst 34580 (blue). Black lines originating from a central point in the nucleus to the tips of the dendrites indicate the measured lengths of each dendrite.

Melanocytes were not counted or measured if the full length of a dendrite was not visible. The results were presented as average number of dendrites per melanocyte and average length of dendrite ( $\mu\text{m}$ ) per melanocyte.

## 2.10 Scratch assay

A scratch assay is used to observe and measure the migration of cells into an area not populated by other cells. It is commonly used in wound healing models since it is a method to simulate a wounded area (Liang *et al.*, 2007). In this project, we wanted to measure the migration of the cells in the different ratios and determine which ratio would provide a better wound healing outcome and the involvement of the melanocytes during wound closure. The cells, as described in Table 2.3, were pre-mixed in 1.5 ml microfuge tubes and seeded onto a 35 mm<sup>2</sup> glass coverslip (placed in a 35 mm tissue

culture dish) overnight. The cells were not allowed to overflow from the coverslip into the tissue culture dish, thereby creating a difference in the confluency level. The scratch was made in the centre of the coverslip with a 1 ml blue micropipette tip, with the aid of a ruler to ensure a consistent scratch (Fig. 2.2). Thereafter, the dishes were washed gently with medium to remove debris and floating cells. Medium was added to the dishes before images were taken on the light microscope (Olympus, Japan) with the Powershot S50 camera (Canon, Japan) at 100 x magnification. These are the “control” images ( $t=0$ ). Images were then taken at subsequent time points (4, 8, 24 and 48 h) before cultures were fixed for ICC to be performed (described in Chapter 2.8).



**Figure 2.2. Diagram depicting the scratch assay**

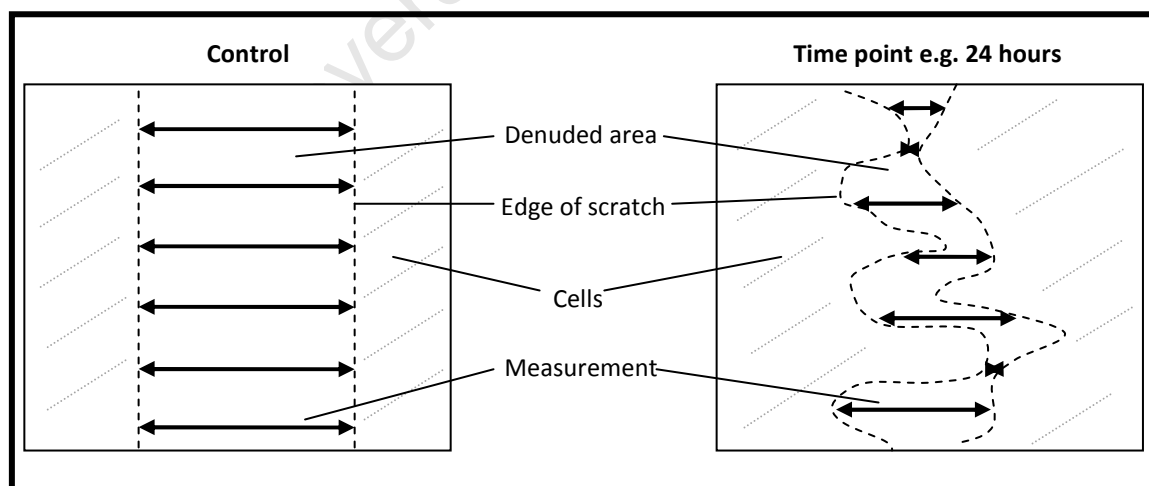
Subsequent ICC images were taken with the AxioVert 200M fluorescence microscope (Carl Zeiss Microscopy, Germany), AxioCam HRm camera (Carl Zeiss Microscopy, Germany) and the AxioVision version 4.8 software (Carl Zeiss Microscopy, Germany).

The percentage of Ki-67 positive melanocytes at the wound edge was calculated (Equation 1) from 10 random fields of view per time point. The rate of wound closure into the denuded area was measured from the images taken on the light microscope using Image J (version 1.43u; National Institutes of Health, USA). Briefly, 6-10 measurements were taken from at least 2 separate fields of view per time point. The average of these measurements was then compared to the average of the “control” images which were taken immediately after the scratch was performed (Fig. 2.3). The results are presented as a percentage of wound closure over a period of 0, 4, 8 and 24 h.

#### **Equation 1**

***Percentage of Ki – 67 postive melanocytes =***

$$\frac{\text{Number of Ki – 67 positive melanocytes}}{\text{Total number of melanocytes}} \times 100$$



**Figure 2.3. Diagram to show how measurements were performed to determine rate of wound closure**



## **2.11 Statistical analysis**

All data were presented as mean $\pm$ SEM. One way analysis of variance (ANOVA) with Bonferroni *post hoc* test was used to determine the significance. A value of  $P < 0.05$  was considered significant. Data was analysed using two way ANOVA with significance set at  $P < 0.05$  for the scratch assays at different time points. All the graphs and statistics were performed using GraphPad Prism (Version 5.01, USA).

## **2.12 Ethics approval**

Ethics approval was obtained for this project from the Faculty of Health Sciences Research Ethics Committee at the University of Cape Town (REC REF: 493/2009).

## CHAPTER 3: RESULTS AND DISCUSSION

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### 3.1 Cell culture

#### *3.1.1 Optimising the mono- and co-culture growth of human melanocytes and keratinocytes*

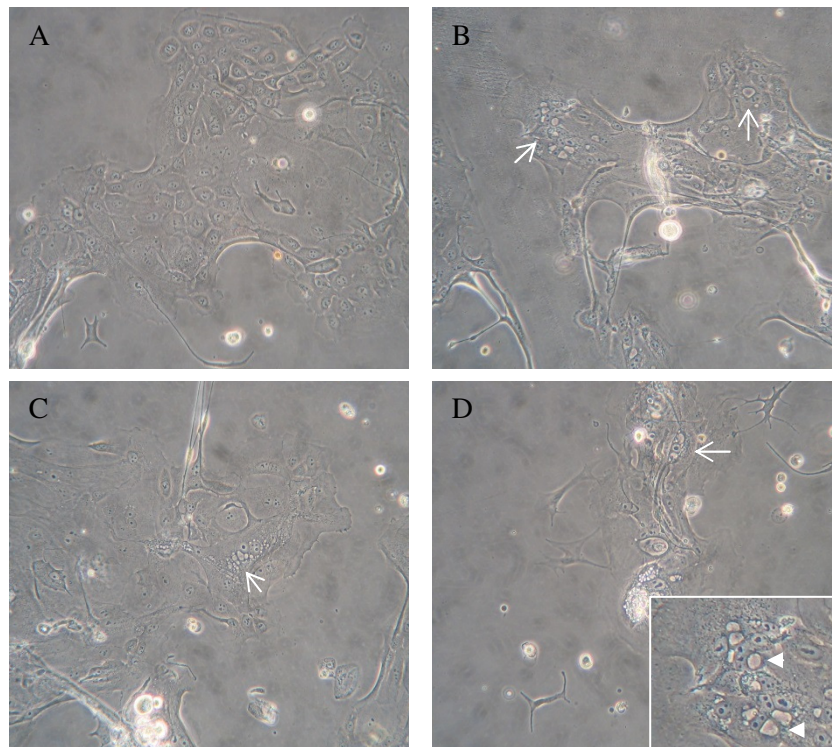
The aims of this project were to promote melanogenesis in an *in vitro* culture system using 3 different ratios of keratinocytes to melanocytes. This was followed by an investigation into which of the ratios would produce an improved clinical outcome with respect to pigmentation and wound healing. Melanocytes and keratinocytes were isolated and cultured from human skin samples. Briefly, the skin samples were ethically obtained from circumcisions and plastic surgery (local hospitals, Cape Town). All the ethical considerations related to the project were approved by our institution (Human Research Ethics Committee of University of Cape Town, Reference no. 493/2009). Two methods were performed to isolate primary melanocytes and keratinocytes (Table 3.1).

Method 1 yielded very few melanocytes and no keratinocytes after 3 days of culture. This poor yield was unexpected as Minwalla *et al.* (2001) had previously used this method successfully. However, it should be noted the difference was that their method was performed by incubating the samples in trypsin at 37°C for 2 h, whereas in this study, the samples were incubated at 4°C overnight, which was used in our laboratory to generate maximum yield. Another possibility for the reduced yield could be that the

processed skin samples used were not small enough. Reducing the size of the samples could therefore be a possible solution. In contrast, method 2 produced both melanocytes and keratinocytes from the skin samples. However, the keratinocytes obtained, struggled to be expanded to further passages as they either did not re-adhere to new Biocoat flasks, or the adherent cells became vacuolated after 2 days (Fig. 3.1).

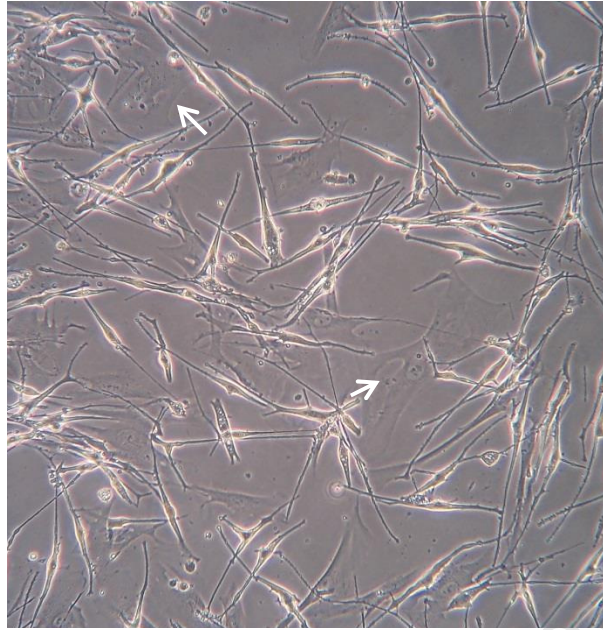
**Table 3.1. The methods used to isolate primary melanocyte and keratinocytes from skin samples.**

| <b><u>METHOD 1</u></b>   | <b><u>METHOD 2</u></b>   |
|--|--|
| Cut samples into 3-5 mm strips   | Cut samples into 3-5 mm strips   |
| Place into <b>0.25% trypsin in PBS</b>   | Place in <b>0.5 mg/ml dispase II (Sigma-Aldrich, USA) in Hank's buffered saline solution</b>   |
| Incubate at 4°C overnight  | Incubate at 4°C overnight  |
| Remove epidermis from dermis   | Remove epidermis from dermis   |
| <b>Transfer epidermis to 5ml, 10% FCS to inactivate trypsin</b>  | <b>Manually disaggregate epidermis</b> into smaller pieces   |
| <b>Manually disaggregate epidermis</b> into smaller pieces   | <b>Transfer disaggregated epidermis into 5 ml, 0.25% trypsin/ 0.05% EDTA</b>   |
| <b>Transfer disaggregated epidermis to clean tubes</b>   | <b>Incubate at 37°C for maximum of 15 min with gentle agitation every 5 min</b>  |
| Centrifuge at 2500 rpm for 2 min   | <b>When solution becomes murky, add FCS to inactivate trypsin</b>  |
| Resuspend pellet in 1 ml Greens (Table 2.2)  | <b>Use 1 ml micropipette tip, with approximately 3 mm of the end cut off, to triturate the solution to get most of the epidermal cells into suspension</b> |
| Transfer 500 µl into a 25 cm <sup>2</sup> collagen I coated Biocoat flasks (BD Biosciences, USA)           | Centrifuge at 2000 rpm for 5 min   |
| Transfer the rest into a 6 cm diameter (6 cm) tissue culture grade petri-dish with FETI medium (Table 2.1) | Resuspend pellet in 1 ml Greens (Table 2.2)  |
|  | Transfer 500 µl into collagen I coated Biocoat flasks  |
|  | Transfer the rest into a 6 cm tissue culture grade petri-dish with FETI medium (Table 2.1)   |

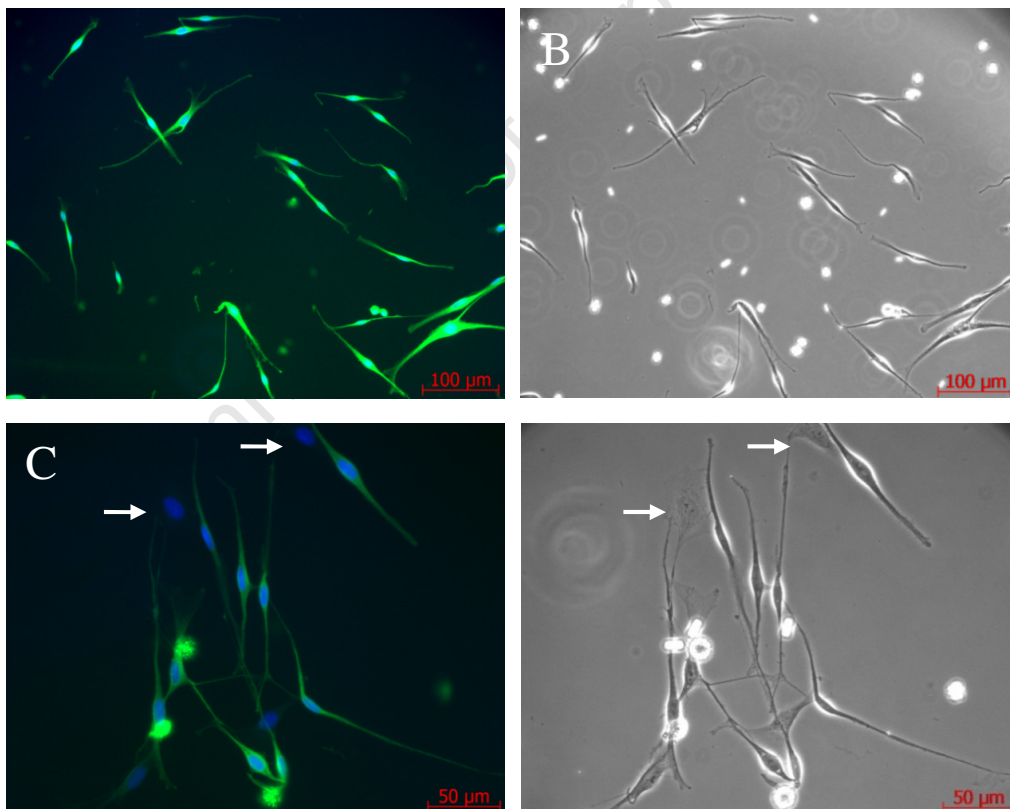


**Figure 3.1. Phase contrast images of primary keratinocytes in culture. A) Healthy keratinocytes, B-D) Keratinocytes that were trypsinised and cultured onto a new Biocoat flask appears to be vacuolated (white arrows). Magnification: 200X (No scale bar was included due to digital resizing). Inset in D shows a higher magnification of the vacuoles.**

As the second method yielded better results, it was further optimised for the isolation of melanocytes, by passing the cell suspension through a 70  $\mu$ M cell strainer (BD Biosciences, USA) before centrifuging. This step was included as some larger pieces of epidermis were not disaggregated into a single cell suspension following the 15 min enzymatic procedure. Geneticin (100  $\mu$ g/ $\mu$ l), which targets the more proliferative cells in a culture, was also added to the primary melanocyte cultures after 5 days of culture to remove the morphologically distinct contaminating fibroblast cells (Fig. 3.2, arrows), resulting in a pure population of primary melanocytes (Fig. 3.3) (Costin *et al.*, 2003).



**Figure 3.2. Human melanocyte culture derived from human skin samples. Evidence of fibroblast contamination can be observed (white arrows). Magnification: 200 X.**

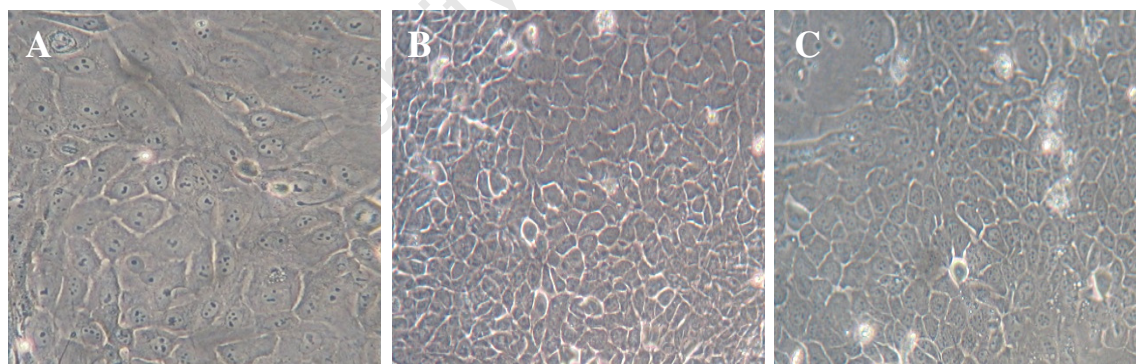


**Figure 3.3. Culture of primary melanocytes. A and C) Melanocytes stained with the melanocyte-specific melanosomal marker, MART-1, green; and nuclear stain, Hoechst 34580, blue. B and D) Phase. A and B) Magnification: 200 X. Scale bar = 100 µm, C and D) Magnification: 400 X. Scale bar = 50 µm. White arrows indicate fibroblast contamination in the culture.**



To confirm the culture purity, cells were stained with the melanocyte-specific marker, MART-1, Fig. 3.3A and C, and compared to the unstained phase micrographs (Fig. 3.3B and D). At higher magnification on phase contrast, it could be observed that some fibroblasts were not completely removed from the melanocyte cultures following the geneticin treatment (Fig 3.3C and D, white arrows).

Despite numerous attempts at propagating the primary keratinocyte cultures, we were unsuccessful. This led to the decision to rather use a human keratinocyte-derived cell line (HaCaTs, a gift from Prof Fusenig, Germany) for all future experiments (Boukamp *et al.*, 1988). HaCaTs are non-tumorigenic, spontaneously immortalised cells, which behave similarly to normal human keratinocytes (Fig. 3.4A). The cells were cultured in DMEM (Fig. 3.4B); however, this was changed to Greens medium (Fig. 3.4C) when the cells were used in the co-cultures with the primary melanocytes.



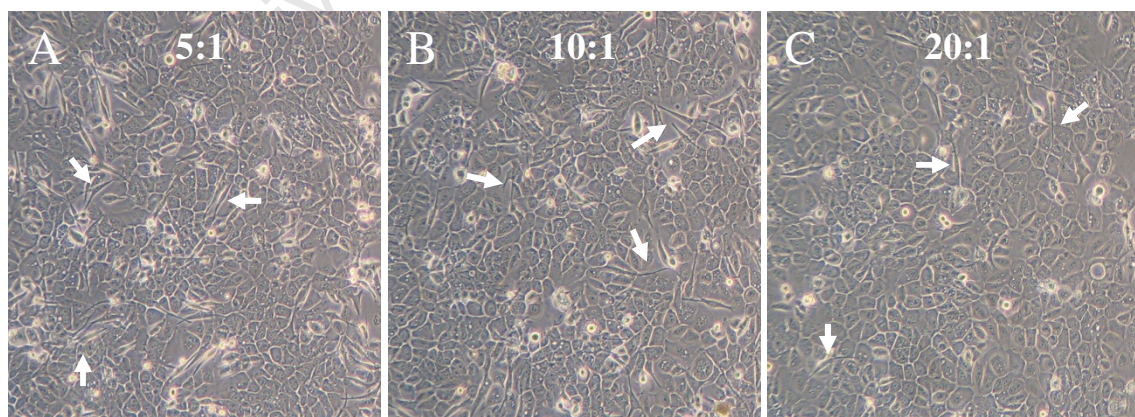
**Figure 3.4. Primary keratinocyte cultures (A) compared to the human keratinocyte cell line, HaCaTs, cultured in B) DMEM and C) Greens medium. Magnification: 200 X**

The cell morphology was similar between the 2 media and no abnormalities were observed when the keratinocytes were cultured in the Greens medium. The cells exhibited typical cobblestone-like appearance and were passaged every 3 days due to

their high proliferative ability (Boukamp *et al.*, 1988, Wang *et al.*, 2012). We did not test the proliferation rate of the cells in the different media, but we speculate that they are similar due to visual observations. However, future considerations could include growth curve assays of the cells in DMEM and Greens. It should be noted, however, that even though HaCaTs are similar to primary keratinocytes, the immortalised cells may behave differently and the data generated from these cell lines could differ to the *in vivo* environment. HaCaTs will be referred to as keratinocytes (Kc) for the rest of this thesis.

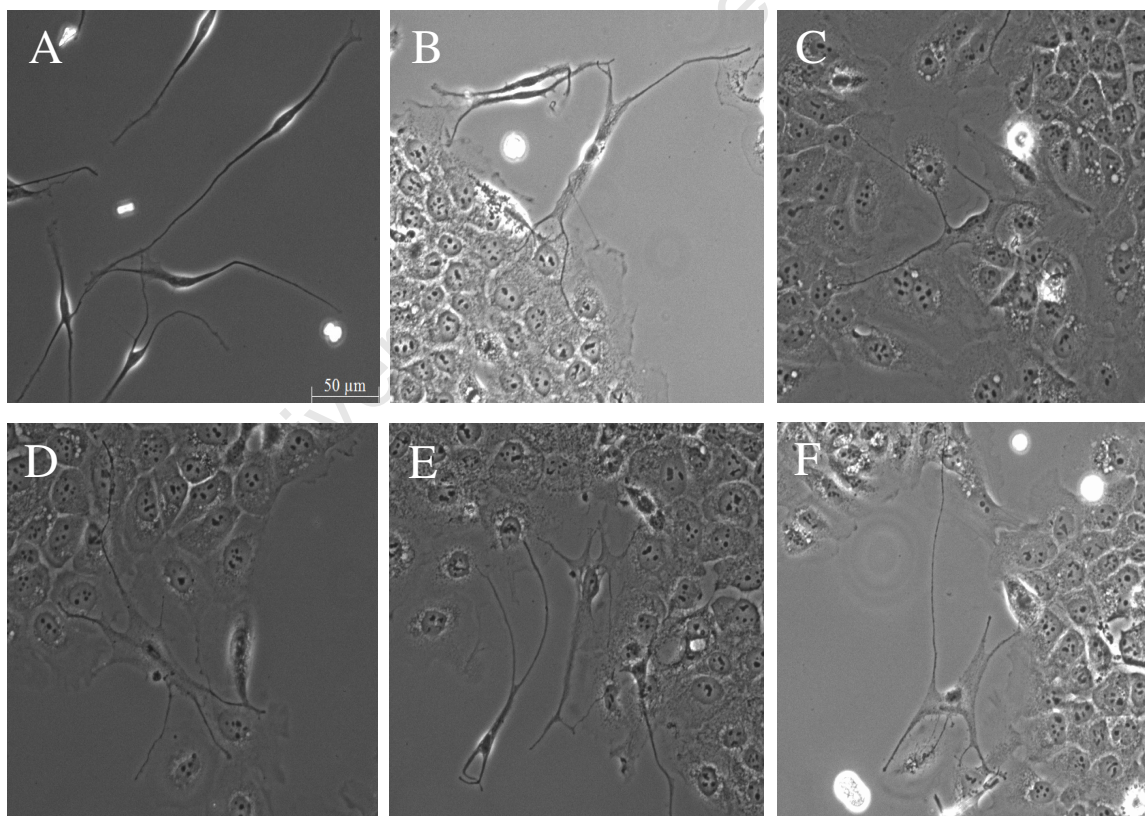
### 3.1.2 Co-culture of melanocytes and keratinocytes

Once the primary melanocytes were successfully isolated and propagated, co-culture experiments in different ratios were set up with the keratinocytes (Chapter 2, Tables 2.3-2.5). Figure 3.5 shows the co-cultures at ratios of keratinocytes to melanocytes of 5:1, 10:1 and 20:1.



**Figure 3.5. Phase contrast images of melanocyte (arrows) and keratinocyte co-cultures seeded at different ratios. A) 5:1, B) 10:1 and C) 20:1. Magnification: 100 X**

Expectedly, due to their individualistic nature, islands of melanocytes were not observed, indicating that the melanocytes were randomly interspersed between the keratinocytes. This showed that the melanocytes and keratinocytes were thoroughly mixed prior to seeding onto the culture dishes. Melanocytes, in mono-cultures have been previously shown to display either a bi- or tripolar phenotype, whereas in co-cultures they display a poly-dendritic morphology (Herlyn *et al.*, 2000, Hsu *et al.*, 2002). This was confirmed in our work through observation of the cells in mono-cultures (Fig. 3.6A) and co-cultures (Fig. 3.6B-F).



**Figure 3.6. Morphology of melanocytes in A) mono-culture and B-F) co-culture. Melanocytes in mono-culture are bi- and tripolar, however in co-cultures with keratinocytes, melanocytes tend to display a poly-dendritic phenotype. Magnification: 200 X. Scale bar = 50 μm.**

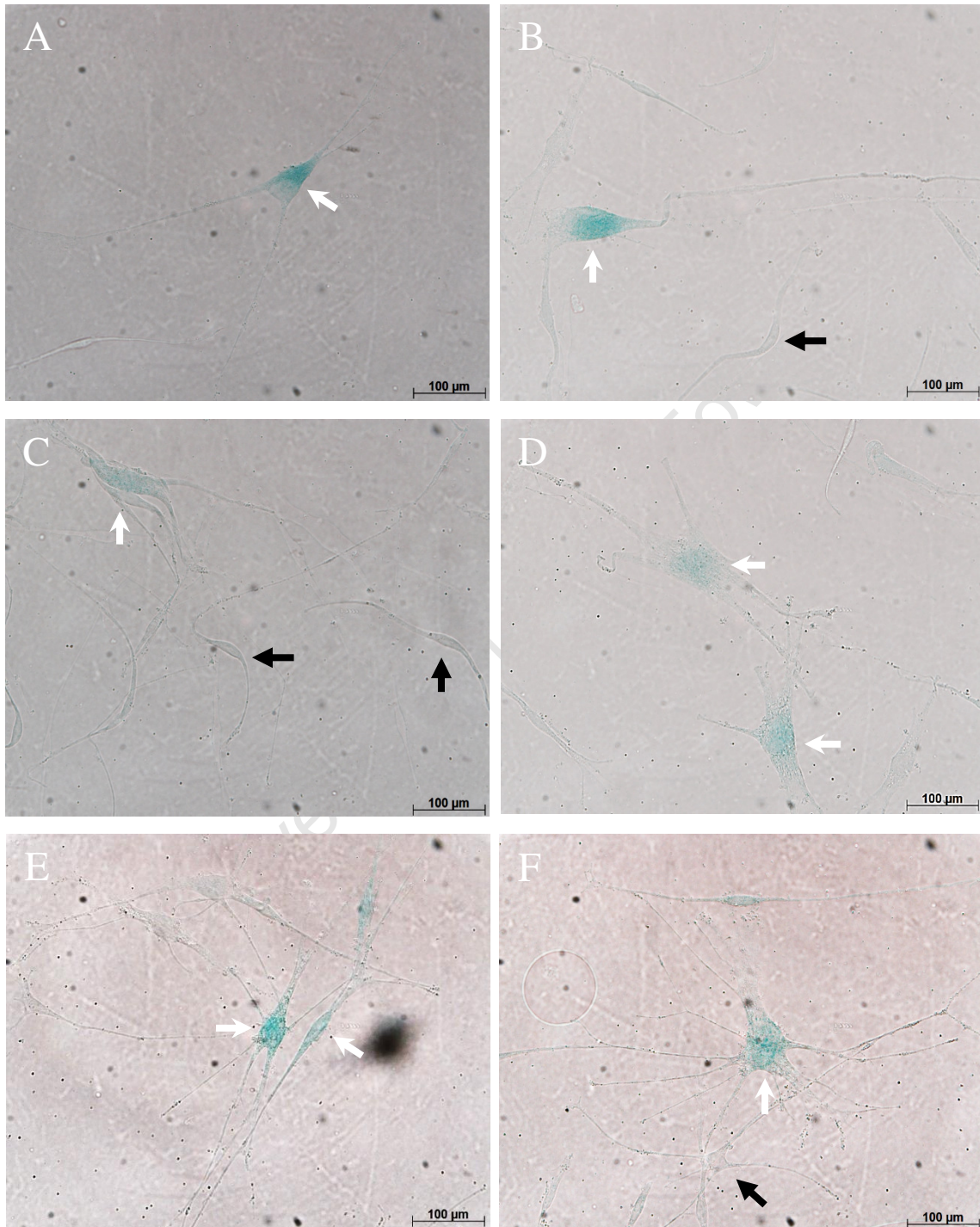


Similarly, melanocytes co-cultured with keratinocytes, displayed a poly-dendritic phenotype, similar to that observed *in vivo*, suggesting that the environment in which melanocytes are found plays an important role with regard to their phenotype (Hsu *et al.*, 2002). Melanocytes in mono-cultures express several antigens, such as  $\beta 3$  integrin subunit and melanotransferrin, that are not typically expressed *in vivo* (Valyi-Nagy *et al.*, 1993). Interestingly, when melanocytes are co-cultured with keratinocytes, these antigens that were previously present are lost (Hsu *et al.*, 2002).

### **3.2 Senescence-associated beta-galactosidase (SA- $\beta$ gal) assay**

We observed that a percentage of the melanocytes in the cultures exhibited a different morphology. The melanocytes appeared to be flattened, i.e. no refractive ring surrounding the cell body; or, exhibited a “spider-like” appearance. The morphology of a cell can often provide an indication of the state of the cell. Cells that have become large and flat, or multi-nucleated often indicate senescence (Kuilman *et al.*, 2010). Senescence is an irreversible process whereby cells lose the ability to proliferate (Debacq-Chainiaux *et al.*, 2009, Cairney *et al.*, 2012, Handayaningsih *et al.*, 2012). Cells undergoing senescence can be detected by the SA- $\beta$ gal assay (Chapter 2.3). SA- $\beta$ gal positive cells will have a “bluish” tinge in the cytoplasm following the staining procedure. Melanocytes were grown in mono- (Fig. 3.7) and co-cultures with keratinocytes; and, as observed in Figure 3.7D-F, certain melanocytes were larger and poly-dendritic. These cells were positive for  $\beta$ gal and hence, senescent. Several of the classic bi- and tripolar melanocytic phenotypes were positive for  $\beta$ gal activity (Fig.

3.7A-C, E). Surprisingly, these  $\beta$ gal positive cells did not display the typical large and flat morphology of senescent cells.



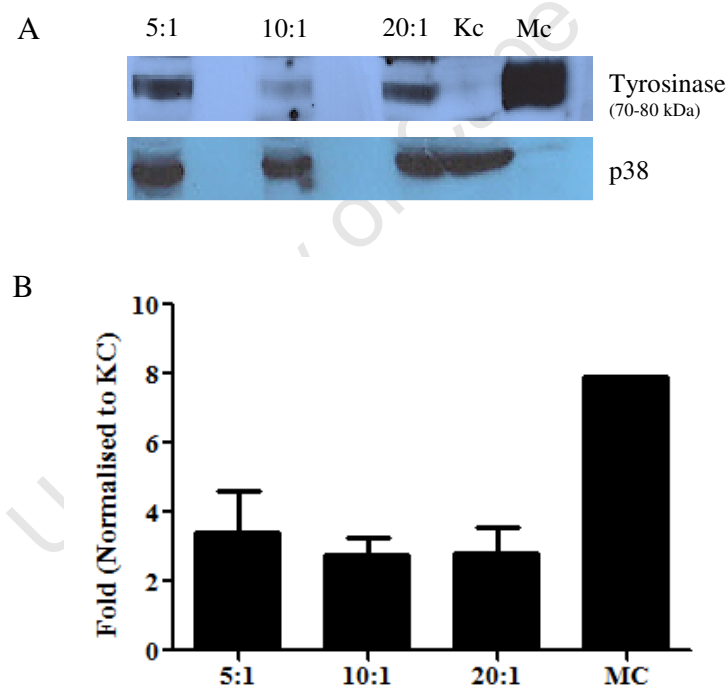
**Figure 3.7. SA- $\beta$ gal staining of melanocyte mono-cultures. Melanocytes with the typical bipolar and tripolar phenotype (A-C) as well as those that are large and poly-dendritic (D-F) are positive for the  $\beta$ gal activity. White arrows indicate senescent cells. Black arrows indicate non-senescent cells. Magnification: 400 X. Scale bar = 100  $\mu$ m.**

Senescent melanocytes were rarely found in the co-cultures (data not shown). A reason for this could be that the keratinocytes were secreting growth factors that prevented senescence, such as endothelin-1 (Hara *et al.*, 1995). It appears that melanocytes rarely underwent senescence; however, it was difficult to detect senescence in the co-cultures. In future, it may be a suggestion to use the fluorescence method of detecting SA- $\beta$ gal activity. Cells can be incubated with C<sub>12</sub>FDG, a  $\beta$ gal substrate, which becomes fluorescent following cleavage by  $\beta$ gal. This fluorescence detection is more sensitive and quantitative (Debacq-Chainiaux *et al.*, 2009).

### 3.3 Tyrosinase protein expression

Tyrosinase, a key enzyme in the formation of melanin, is a marker of differentiated melanocytes (Opdecamp *et al.*, 1997). Furthermore, it is a key enzyme in melanogenesis as it oxidises DOPA to DOPAquinone and following a number of further biochemical steps, results in the formation of melanin (Fig. 1.4). The other important aspect of tyrosinase is that its expression is melanocyte specific. Tyrosinase expression was analysed through western blotting techniques where the total cellular protein was extracted after 6 days of co-culture. Figure 3.8A shows a representative blot of 2 independent experiments. The negative control was a pure culture of human keratinocytes (Kc) and as expected, no band was observed. The primary human melanocyte culture (Mc) was used as the positive control for tyrosinase expression (70-80 kDa). The detection of the p38 protein (38 kDa) was used as an internal loading control in all experiments. Even though less protein was loaded for Mc (5  $\mu$ g), it still shows a remarkably high tyrosinase expression. The other samples were loaded with 80

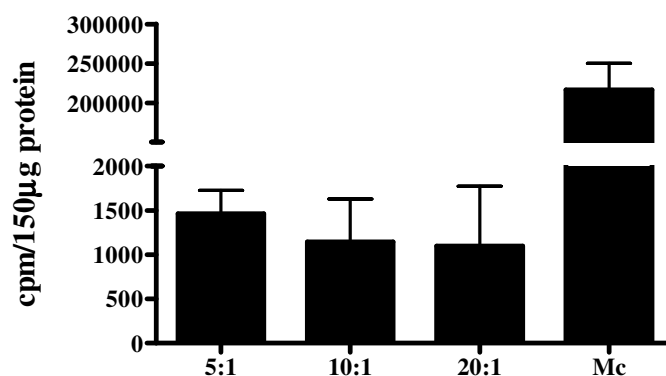
µg of protein. The 3 ratios of keratinocytes: melanocytes were all positive for the tyrosinase protein expression with the 5:1 ratio showing a higher expression than the other ratios. However, the densitometric analysis shows no difference between any of the ratios (Fig. 3.8B). Densitometry showed a 3.4, 2.7 and 2.8 fold increase in the 5:1, 10:1 and 20:1 ratios, respectively. The melanocyte control had the highest fold increase (7.9). Therefore, despite visually seeming different in the western blots, the densitometric quantifications emphasises the need for absolute quantification.



**Figure 3.8. Western blot of tyrosinase and p38. A)** Western blot confirms the presence of tyrosinase in the 3 different ratios of keratinocytes to melanocytes – 5:1, 10:1 and 20:1. Kc = keratinocyte only control, whereas Mc = primary human melanocyte. This is a representative of 2 independent experiments. **B)** Densitometry showing fold increase of tyrosinase expression when normalised to KC.

### 3.4 Tyrosinase enzymatic activity

Despite the tyrosinase expression being non-significant between the different ratios, the question of tyrosinase enzymatic activity between the different ratios was investigated. This was done in order to find out if it correlated to tyrosinase protein expression and was conducted by way of a tyrosinase activity assay. The level of enzyme activity was quantified as counts per minute (cpm) of incorporated radioactive tyrosine in 150 µg of total protein. The data was normalised to the keratinocyte control. Figure 3.9 shows that similar amounts of enzyme activity were found between the 3 ratios. Unsurprisingly, the tyrosinase activity assay correlated with the tyrosinase western blot expression in that none of the ratios showed significance. The 5:1, 10:1 and 20:1 ratios showed a mean of  $1461.7 \pm 262.4$ ,  $1146.2 \pm 483.1$  and  $1097.2 \pm 677.3$  cpm, respectively. The melanocyte (Mc) control exhibited the highest level of tyrosinase activity at  $2165069 \pm 33794.1$  cpm. Hence, the enzyme activity in the ratios appeared to be considerably lower than the control. As tyrosinase activity was detected in the ratios, it could be concluded that the melanocytes were actively synthesising melanin. However, the level of activity of melanogenesis in the melanocytes was not affected by the keratinocyte numbers in the different ratios.



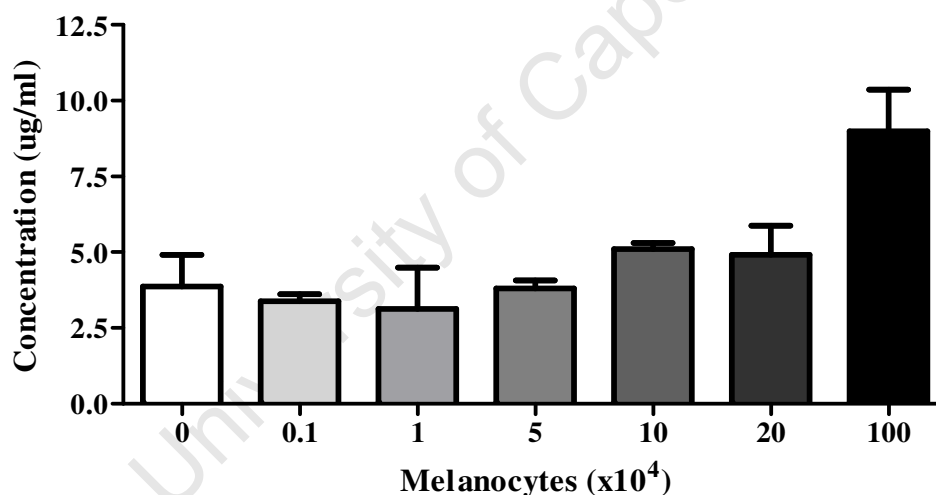
**Figure 3.9.** Enzyme activity of tyrosinase between the different ratios. Data is normalised to the keratinocyte control. The ratios of keratinocytes to melanocytes – 5:1, 10:1 and 20:1. Melanocyte (Mc) represents the positive control. Data is presented as mean±SEM, n=3.

### 3.5 Melanin assay

Despite no difference being observed in the level of tyrosinase enzyme activity, we next wanted to determine if there was a difference in the amount of melanin produced between the ratios. Regardless of numerous modifications to the protocol, several methodological problems were encountered. Firstly, the protocol involved lysing the cells and creating a pellet through centrifugation. However, we were unable to acquire a pellet after the centrifuge step. Secondly, there was debris in the supernatant which made the pipetting into 96-well plates for triplicate readings, difficult and inconsistent. This may have been due to the amount of keratinocytes in the samples as the melanocyte only control, showed no such characteristic. Therefore, further modifications were needed to quantify the amount of melanin in the co-cultures.

One option was a “spiking” experiment to determine the sensitivity of this assay in detecting very low quantities of melanin. For this, up to  $2 \times 10^5$  melanocytes were added

to  $1 \times 10^6$  keratinocytes. Using an established protocol (Oka *et al.*, 1996), our results confirmed that this assay was not sensitive enough to detect the low amount of melanin produced in the co-cultures (Fig. 3.10). The experiment shows the background reading of this assay is  $\pm 4 \mu\text{g/ml}$ . Only the pure culture of melanocytes showed an increase in the concentration of melanin ( $\pm 9 \mu\text{g/ml}$ ), whereas the spiking of the melanocytes showed similar levels of melanin to the keratinocyte only control ( $0 \times 10^4$  melanocytes). This re-emphasised the difficulty of reading melanin in a co-culture system and highlighted the need for an alternative method to quantify and determine the pigimentary phenotype of the 3 co-cultures.



**Figure 3.10.** Melanocyte "spiking" assay. Melanocytes ranging from  $0.1$  to  $20 \times 10^4$  were added to  $1 \times 10^6$  keratinocytes. The pure keratinocyte culture ( $0$  melanocytes added) is the negative control, whereas a pure melanocyte culture ( $100 \times 10^4$ ) is used as a positive control.

In further support, many of the articles regarding melanin assays have been performed primarily on pure melanocyte/melanoma cultures or melanosome uptake experiments by the keratinocytes (John *et al.*, 2005, Singh *et al.*, 2010). Lei *et al.* (2002) however was

the one group that used immortalised murine cell lines with a keratinocyte to melanocyte ratio of 5:1. However, after 6 days of co-culture, they found that the ratio became 2.5:1. Furthermore, they did not centrifuge the samples after the cells were lysed with NaOH. This, and including the higher ratio of melanocytes in their samples, probably allowed them to acquire reproducible, detectable melanin values.

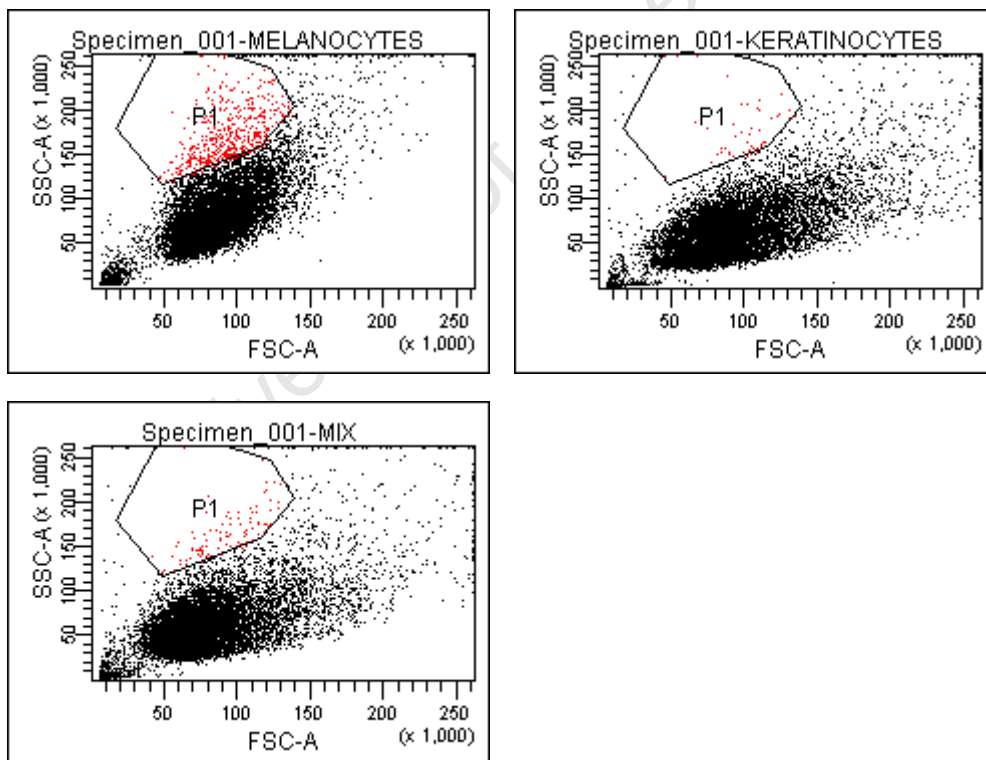
The next option was to culture the cells in different ratios and separate the melanocytes from the keratinocytes in order to repeat the western blot, tyrosinase assay and melanin assay analyses on each cell type. The rationale for this was that post-separation, a western blot would inform us if tyrosinase expression was found in the keratinocytes. If it was, the tyrosinase enzyme activity assay would determine if the enzyme was still active even after the transfer of melanosomes from the melanocytes. It was previously shown that tyrosinase activity was present in the melanosomes that were secreted into the culture medium by primary mouse melanocytes (Costin *et al.*, 2003). Simultaneously, in light of the limitations to the melanin assay mentioned above, having an isolated population of melanocytes would allow us to perform the melanin assay without the interference of the keratinocytes in the samples.

To separate the 2 cell types, we initially tried to “differentially” trypsinise the melanocytes from the co-cultures, as we knew from experience, that melanocytes lifted more readily than the keratinocytes. This trypsinisation method was no different to the normal trypsinisation method of the cells (Chapter 2.1.2). We confirmed the observations of Berens *et al.* (2005), in that this method was unsuccessful as not all of the melanocytes could be lifted. One possible reason for this could be that the



melanocytes in direct contact with the keratinocytes form very tight junctions with the keratinocytes (Tang *et al.*, 1994, Haass *et al.*, 2004).

We next decided to sort the cells by size and granularity using fluorescence-activated cell sorting (FACS). The rationale behind this was that keratinocytes are larger than melanocytes, and that melanocytes are more granular than the keratinocytes due to the melanin/melanosomes in the cells. Surprisingly, we discovered that the pure melanocyte and keratinocyte cultures are very similar in size and granularity post-trypsinisation (Fig. 3.11).

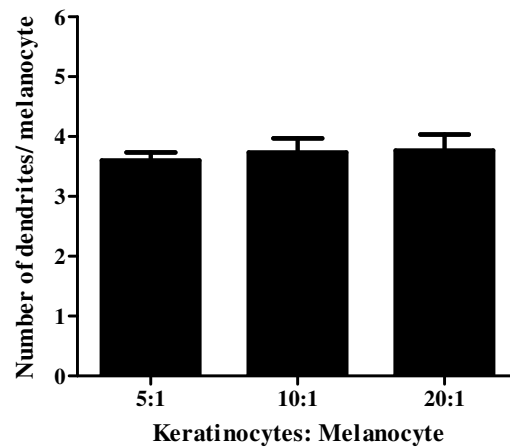


**Figure 3.11.** Forward scatter (FSC) and side scatter (SSC) of human melanocytes and keratinocytes, and a “mix” of melanocytes and keratinocytes. FSC relates to size of cells and SSC relates to the granularity of the cell. P1 refers to the population of cells with increased granularity.

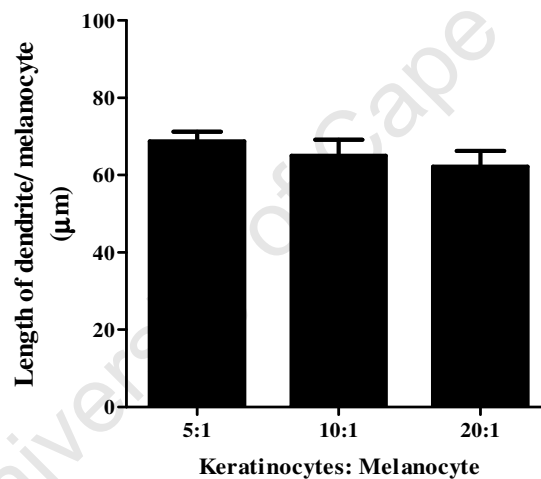
A further approach was to stain one cell type with a cell-specific FACS-related antibody and proceed with cell sorting. The antibodies used were either the melanocyte-specific MART-1/MELAN-A, which targets the stage 1 and 2 melanosomes in the melanocytes (Delevoye *et al.*, 2011); or a keratinocyte-specific cytokeratin antibody. During the FACS staining procedure, it was noticed that many cells were lost during the washing steps and the cells did not readily take up the antibody. This approach, due to time constraints was therefore, abandoned.

### 3.6 Dendricity index

Although the melanin assay was not definitive, it is established that the dendrite number and length of melanocytes correlate with an increase in melanin content and melanosome number (Friedmann *et al.*, 1987). Based on this, we used the dendrite number and length as markers of melanogenesis/differentiated cellular state. The aim was to determine if there was a difference in the morphology of the melanocytes, with respect to i) the number of dendrites per melanocyte, and ii) the length of these dendrites in the co-cultures of the different ratios. The number of dendrites was  $3.6 \pm 0.1$ ,  $3.7 \pm 0.2$  and  $3.8 \pm 0.3$  for 5:1, 10:1 and 20:1, respectively. The results show an average of 4 dendrites per melanocyte for all 3 ratios (Fig. 3.12). The average length of each dendrite for 5:1, 10:1 and 20:1 was  $68.8 \pm 2.4$ ,  $65.1 \pm 4.1$  and  $62.2 \pm 4.1$   $\mu\text{m}$ , respectively (Fig. 3.13).



**Figure 3.12.** Dendricity index showing average number of dendrites per melanocyte in 10 randomly selected fields of view. Data is presented as mean±SEM from 3 independent experiments.



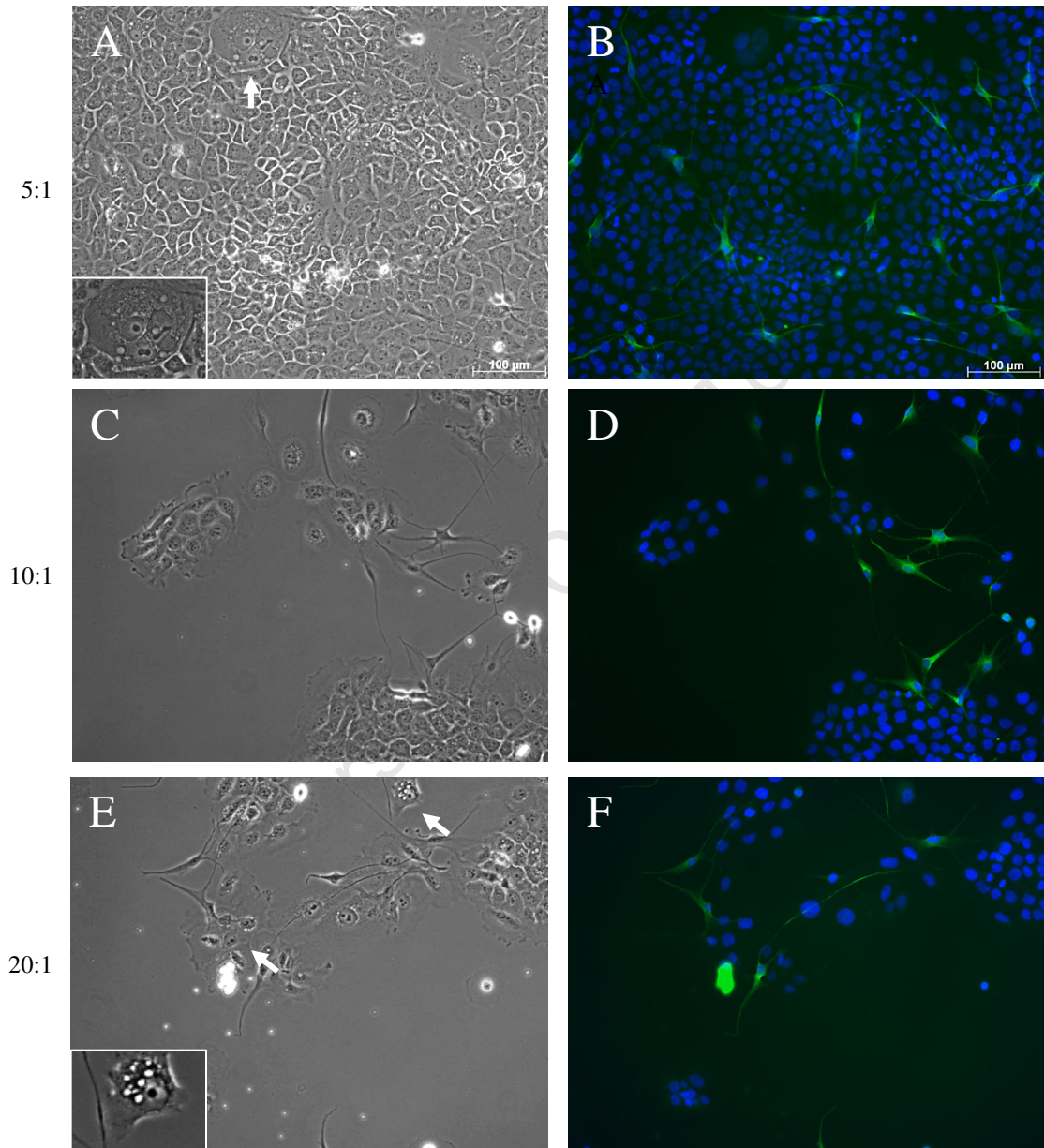
**Figure 3.13.** Dendricity index showing average length of dendrites per melanocyte in 10 randomly selected fields of view. Data is presented as mean±SEM from 3 independent experiments.

In an earlier study, Jungbauer *et al.* (2004) reported that the mean number of dendrites per melanocyte was 2.2. Our results showed a higher dendricity average, possibly as our assays were performed in co-cultures, compared to their mono-culture experiments. In our study, there was no significant difference between the 3 ratios for the number and length of dendrites. This implied that the melanocytes in the ratios produced a similar

quantity of melanin, irrespective of the keratinocyte to melanocyte ratio. This correlated with the fact that tyrosinase activity was not significantly different between the ratios. This further confirms that keratinocytes did not upregulate known factors that enhance melanogenesis such as  $\alpha$ -melanocyte-stimulating hormone, prostaglandin E2 and endothelin-1, which are involved in pigmentation and dendricity of the melanocytes (Hunt *et al.*, 1996, Scott *et al.*, 2007, Hara *et al.*, 1995, Imokawa, 2004).

Microscopic analyses of these immunocytochemically-labelled co-cultures display a slightly different picture. Representative images of the different ratios taken at phase (Fig. 3.14A, C and E), as well as ICC images of MART-1 and Hoechst 34580 (Invitrogen, UK) staining, are shown (Fig. 3.14B, D and F). Vacuoles (white arrows) within the keratinocytes can be observed in some of the cultures, which could be an indication of these cells undergoing senescence or apoptosis. Studies relating to senescence, suggests that cells display typical features, which include cells that are larger in size, irregularly shaped and contain many vacuoles within the cytoplasm (Kang *et al.*, 2000, Soroka *et al.*, 2008). Moreover, cells undergoing apoptosis display a shrinking of the cells, blebbing and vacuoles in the cytoplasm (Saraste *et al.*, 2000, Abbro *et al.*, 2003, Gonzalez-Polo *et al.*, 2005). This appearance of vacuoles could also have been due to the lack of nutrients as HaCaTs are known to be very proliferative; and hence, could have depleted the available nutrients (Boukamp *et al.*, 1988). This assay was set up with a constant melanocyte number, therefore the keratinocyte number increased from  $5 \times 10^4$  to  $2 \times 10^5$ . Because of the high total cell number, we were concerned that 35 mm tissue culture dishes would become too confluent after a period of 4 days of culture. Therefore, the other possibility for the vacuoles in the keratinocytes

could be that the cells were either too confluent (for the 5:1 ratio) or sparse (for the 10:1 and 20:1 ratios) as different sized tissue culture grade dishes were used to culture the cells (Fig. 3.14).



**Figure 3.14. Representative images of the different keratinocyte to melanocyte ratios. A, C and E) Phase contrast; B, D and F) melanocyte-specific MART-1 (green) and Hoechst 34580 (nucleus, blue). White arrows indicate vacuoles observed in the keratinocytes. Magnification: 100 X. Scale bar = 100  $\mu$ m.**

Cells grown in culture have been known to have contact inhibition, where cell growth is arrested, due to the cells being too close to one another (Seluanov *et al.*, 2009). Cells also do not grow in an environment where they are too far apart as they lose the cell-to-cell contact, and therefore, cannot communicate with each other (Rubin *et al.*, 1973). Future dendricity assays could be performed with the same cell numbers and in 35 mm tissue culture dishes, however, with increased volume of medium, or the medium could be changed after 2 days of co-culture. Another suggestion could be to reduce the melanocyte cell number, which will in turn decrease the total cell number and therefore, alleviate the problem of over-confluency.

The data generated thus far, points to the fact that normal melanogenesis takes place in all 3 ratios. Moreover, as there was no difference in the ratios, it can be assumed that the pigimentary phenotype was identical. In summary, the keratinocytes did not affect tyrosinase processing and enzyme activity, and therefore melanogenesis. The keratinocytes also did not affect the morphology of the melanocytes, with regard to the dendricity of the cells. Finally, we determined that the melanin assay was too insensitive to detect the melanin in our co-culture system. A few suggestions to overcome this, is to isolate and quantify melanosomes, or to use more advanced high-performance liquid chromatography techniques (Alaluf *et al.*, 2001, Donois *et al.*, 1998).

### 3.7 Scratch assay

In order to investigate the pigmentary phenotype of the 3 different co-cultures, this project looked at tyrosinase enzyme expression and activity, and the quantification of dendricity. Now that we have shown that all 3 ratios exhibit a similar level of pigmentation, we wanted to determine which of the 3 ratios would provide an optimal wound healing outcome and whether there was, if any, involvement of the melanocytes during wound closure. Hence, scratch assays were performed to observe the migration and proliferation of cells into a wounded area. The percentage of wound closure was determined over a 4 to 48 hour period (Fig. 3.15).

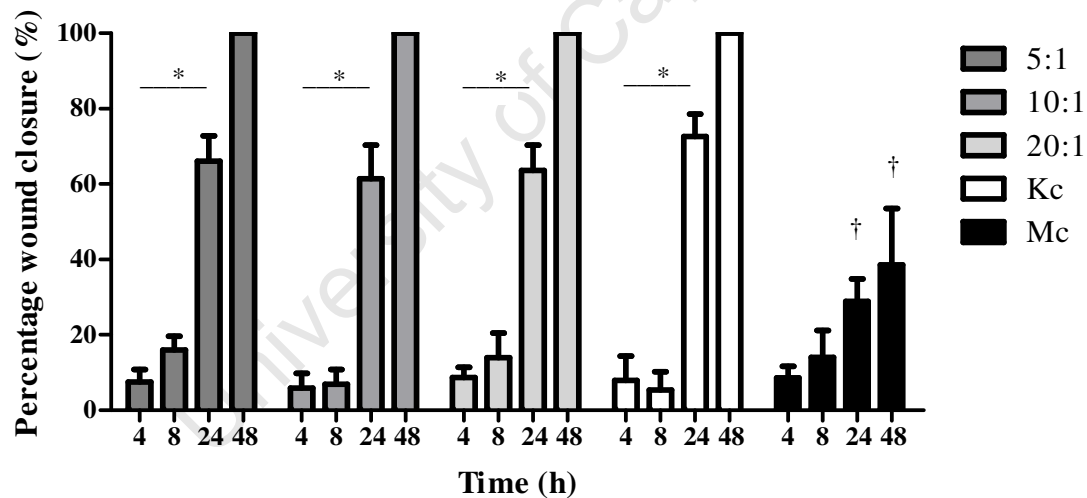
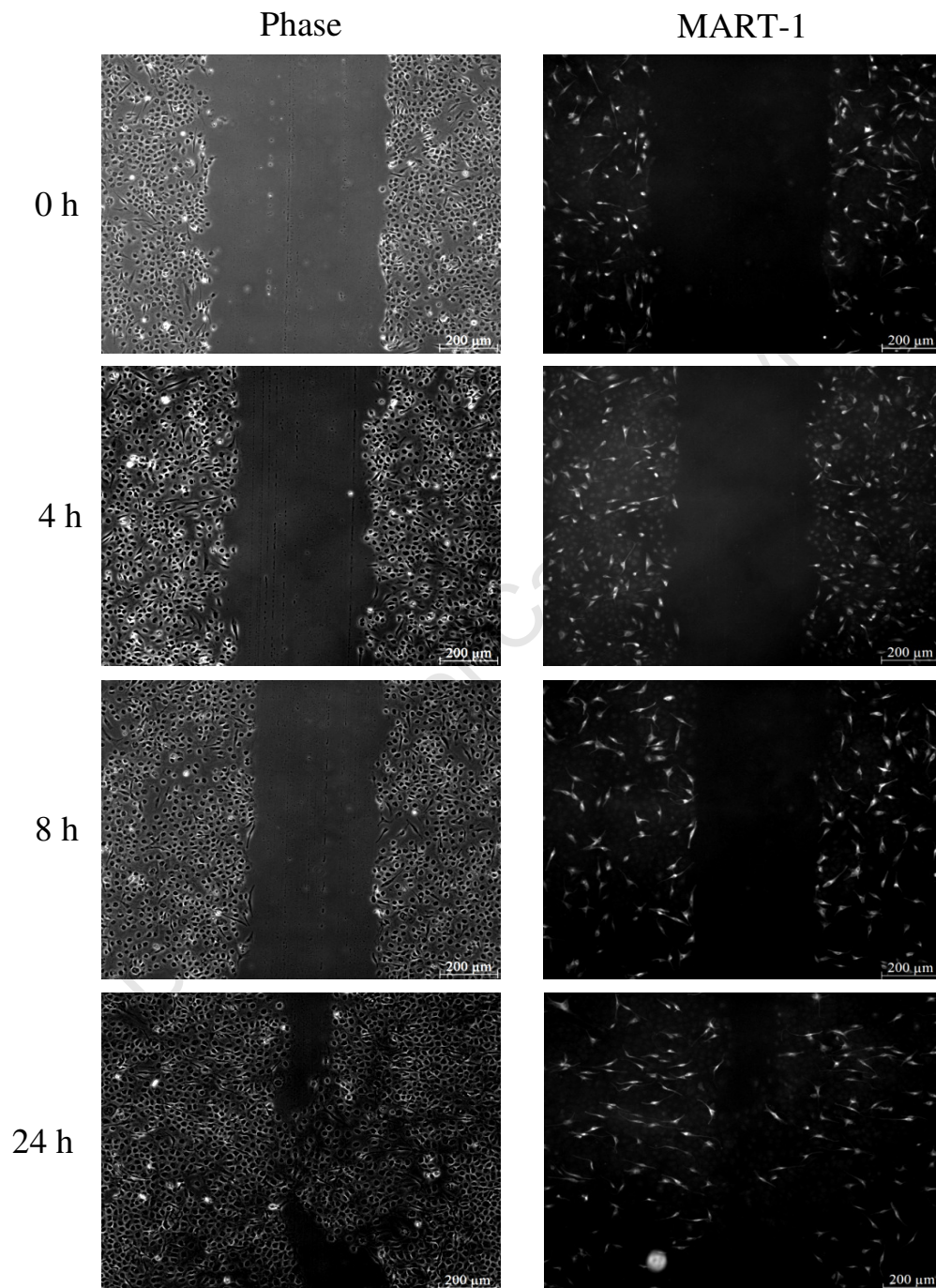


Figure 3.15. Percentage wound closure at 4, 8, 24 and 48 hours after the scratch. When the wound was made (0 h), wound closure was 0 %. Ratios are represented as keratinocytes: melanocyte, Kc = keratinocyte control, Mc = melanocyte control. Data is shown as mean $\pm$ SEM of 3 independent experiments. The percentage wound closure at 24 h time points were compared to the 4 h time points of each ratio or control, \*P<0.05. Each Mc time point was compared to the corresponding time points of the ratios or Kc control, †P<0.001.

Creating the wound on the dish at time 0 h was defined as 0 % and progressive wound closure was normalised as a percentage against the initial wound (0 %). At 4 and 8 h after the scratch was made, wound closure was minimal and no significant differences were observed. The percentage of wound closure ranged from 5.9 to 8.7 % for the 4 h time point, whereas wound closure was 5.4 to 16 % for the 8 h time point between the ratios and the controls. The rate of wound closure occurred more rapidly between 8 h and 24 h after the scratch was performed. At 24 h post-wounding, the rate of closure ranged from 61.4 to 72.6 % between the 3 ratios and the keratinocyte control. These values were significantly different compared to their 4 h time points (\* $P < 0.05$ ). The rate of wound closure of the melanocyte control was 28.9 %. This was significantly different to the 3 ratios and the keratinocyte control ( $\dagger P < 0.001$ ). The results showed that the migration of melanocytes in the mono-cultures was slower than the keratinocytes. However, in the presence of keratinocytes, the rate of migration was increased as the melanocytes were demonstrated to be in the edge of the migrating keratinocytes (Fig. 3.16). This implied that the keratinocytes are the source of migratory and proliferative factors, such as tumor necrosis factor- $\alpha$ , basic fibroblast growth factor and transforming growth factor- $\beta$ , during wound healing (Gauglitz *et al.*, 2012). We, therefore, suggest a theory that keratinocytes signal to the melanocytes to migrate into the wound in the co-cultures. However, in the absence of keratinocytes, there is a lack of signalling molecules to recruit the melanocytes into the wounded area. The rate of wound closure 48 h after the scratch was made, was 100 % for the 5:1, 10:1, 20:1 ratios and the Kc control. The wound closure at 48 h for the Mc control was 38.5 %. This was significantly different to the ratios and Kc control ( $\dagger P < 0.001$ ). This showed that the

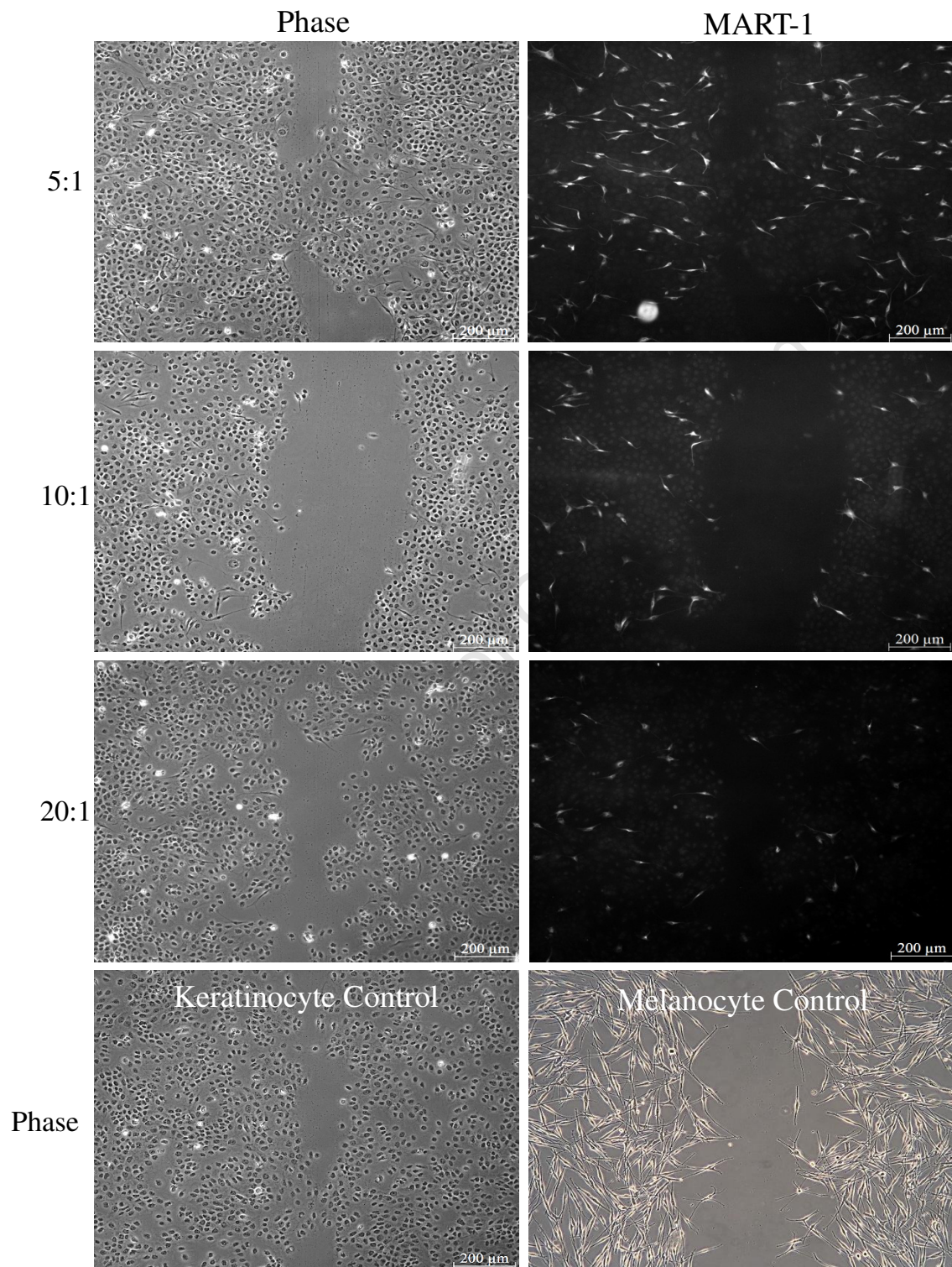


involvement of keratinocytes in wound healing is important as it is involved in complete wound closure (Haase *et al.*, 2003, Patel *et al.*, 2006).



**Figure 3.16.** Scratch assay images in phase contrast microscopy (left panels) and MART-1 staining (right panels) of 5:1 ratio over a period of 24 hours. This is a representative image for all the ratios. The 10:1 and 20:1 ratio scratch assays were not shown as no difference was observed between the 4 and 8 h time points. Magnification: 100 X. Scale bar = 200 μm.

Representative phase and ICC images of the scratch assay at the different time points were shown in Figures 3.16 and 3.17.



**Figure 3.17.** Scratch assay images at 24 h post-scratch, showing the wound closure of the ratios and the keratinocyte and melanocyte controls. Magnification: 100 X. Scale bar = 200 μm.

As observed, wound closure at 4 h and 8 h was minimal. At 8 h, the melanocytes have migrated into the wound and lined up against the border of the keratinocytes. This was similar to previous work in our laboratory (Keswell *et al.*, 2012). Although not shown, it is interesting to speculate that at this stage, the keratinocytes are secreting specific factors in response to injury, such as interleukin-1 (IL-1). IL-1 is secreted by keratinocytes during wound healing where it induces an autocrine response leading to keratinocyte migration and proliferation (Kiwanuka *et al.*, 2012). The effects of IL-1 on melanocytes, however, has a dual role in that, it i) attracts the melanocytes to repopulate the wound with the migrating and proliferating keratinocytes and thereby not lose the ratio of melanocytes to keratinocytes and ii) decreases melanocyte proliferation and melanogenesis *in vitro*, thus keeping the melanocytes in an undifferentiated, migratory state (Swope *et al.*, 1991). Future work could include determining the level of IL-1 RNA and protein expression in the scratch assays.

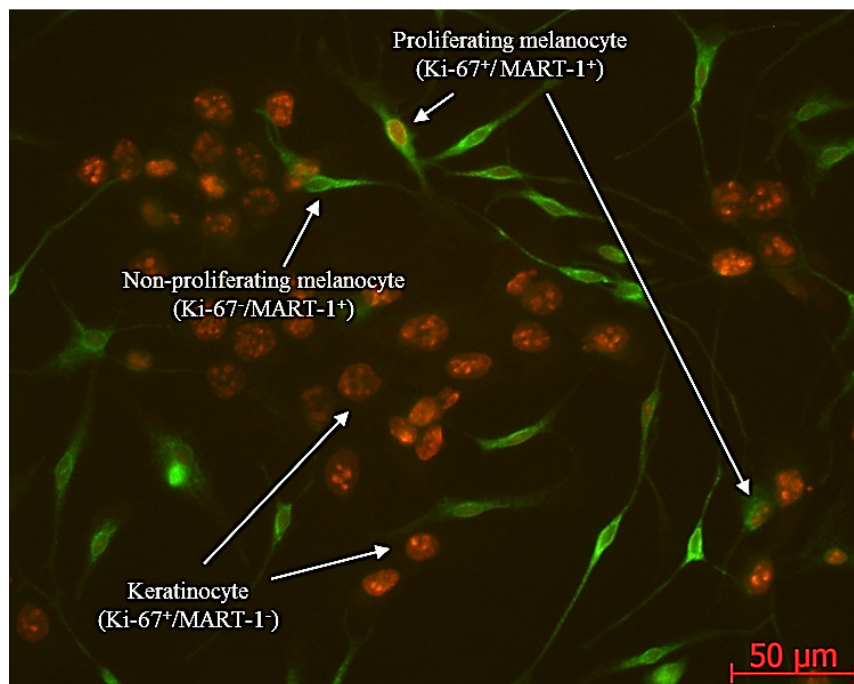
Valyi-Nagy *et al.* (1993) demonstrated that the initial seeding ratio of melanocytes to keratinocytes remain constant, even after several days in co-culture. In contrast, our study showed that the initial seeding ratio does not remain constant. The cells used in this study exhibited different proliferative rates - the HaCaTs are highly proliferative cells with a doubling time of 18-20 h, whereas the primary human melanocytes have a doubling time of 2-3 days (Boukamp *et al.*, 1988, Wang *et al.*, 2012, Valyi-Nagy *et al.*, 1993, Boukamp *et al.*, 1990, Alexaki *et al.*, 2012). In support of our data, Lei *et al.* (2002) in their murine cell culture model showed that the initial seeding ratio changed after several days in co-culture. The dual functionality of IL-1 is interesting as proliferation and migration are mutually exclusive events, therefore, in decreasing

proliferation, it allows for melanocytes to focus on migration (Zheng *et al.*, 2009). Melanocytes at the 24 h time point were either lagging a few cells behind the keratinocyte wave, directly in front of the keratinocytes or both (Fig. 3.17).

This is in contrast to Heath *et al.* (2009), who showed that melanocytes *in vivo* were not present in the migrating epithelial tongue. Furthermore, they noticed that melanocytes were found in the newly epithelialised wound, lagging behind the keratinocytes by 0.45 – 2.85 mm. However, they do not mention if the melanocytes were being “carried” by the keratinocytes as the keratinocytes proliferated and migrated to close the wound; or, whether the melanocytes migrated into the wounded area once it has been fully/partially re-epithelialised. In recent work, our group demonstrated that the melanocytes are able to migrate through tightly bound keratinocytes (Keswell *et al.*, 2012). Moreover, we showed melanocytes migrating into the wounded area, “leading” the keratinocytes. In addition, melanocytes became bipolar with “dendrites protruding into the spaces” (Keswell *et al.*, 2012). Similarly, our scratch assays displayed the same phenotype where many of the melanocytes were bipolar with one arm of the dendrite “pointing” towards the wounded area. A possible reason for this could be that dendrites were a mechanism for finding a path through the keratinocytes. The dendrites would extend and once the pathway has been found, it would then attach to the keratinocytes and both the cell body and the nucleus are pulled through between the tightly packed keratinocytes (Keswell *et al.*, 2012). Alternatively, as suggested by Chadwick *et al.* (2012) that in order to reduce oxidative stressors, a response to wound healing, melanocytes would quickly repopulate the wounded area, to scavenge the free radicals produced by the surrounding cells (Chadwick *et al.*, 2012).



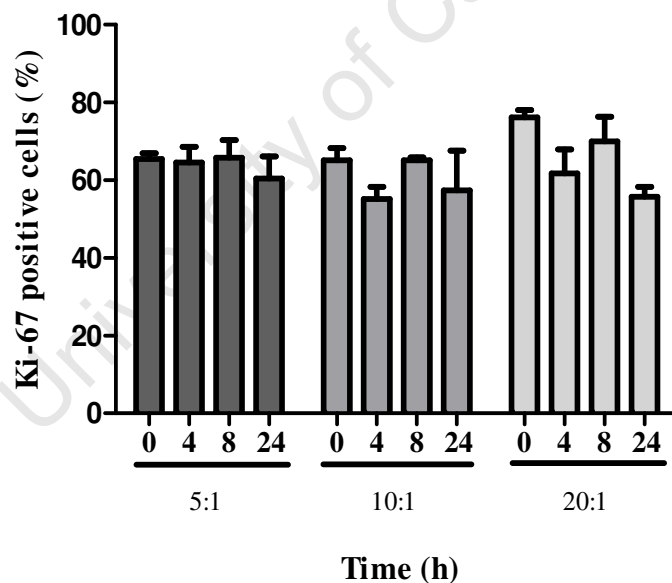
As the proliferation of melanocytes in and around the wound bed is a rare event, the objective was to investigate how these cells were affected in the presence of keratinocytes. To do this, immunocytochemistry was utilised and the co-cultures were stained with MART-1 (melanocyte-specific) and Ki-67 (proliferation marker) antibodies. An example of melanocytes (green, MART-1<sup>+</sup>) that are either proliferating (red, Ki-67<sup>+</sup>) or not proliferating (Ki-67<sup>-</sup>) is shown in Figure 3.18.



**Figure 3.18.** Example of the Ki-67 and MART-1 double staining. Melanocytes are stained green (MART-1<sup>+</sup>). Proliferating melanocytes were green with a red nucleus (Ki-67<sup>+</sup>). Non-proliferating melanocytes do not have a nuclear stain (Ki-67<sup>-</sup>). Keratinocytes are represented by a Ki-67<sup>+</sup> nucleus. Magnification: 400 X. Scale bar = 50 μm.

Quantifying the percentage of proliferating melanocytes in the periphery of the wound over time showed no significant reduction in cell proliferation in any of the ratios used. The percentage of Ki-67 positive melanocytes ranged from 55.2 to 76.2% between 0 and 24 h for all the ratios (Fig. 3.19). In observing the melanocytes close to the wound

edge to determine their involvement during wound healing, no significant differences were found between the 3 ratios or the time points. This suggests that despite one particular cell type (melanocytes) entering the wound bed before the other (keratinocytes), no difference in the rate of proliferation occurs, irrespective of the number of surrounding keratinocytes. This is interesting in light of the fact that one would assume that with increasing keratinocytes in the surrounding environment, more proliferative factors may be released. This suggests therefore, that either the keratinocytes in a “wound-healing” situation down-regulates their own factors, or the melanocytes induce a down-regulatory effect at an autocrine level (on themselves) and/or paracrine level (on the keratinocytes).



**Figure 3.19.** Percentage of proliferating melanocytes close to the wound edge. The number of Ki-67 positive melanocytes was counted and normalised against the total melanocytes in 10 fields of view. Data is represented as mean $\pm$ SEM from 3 independent experiments.

Although the specific mechanism of melanocyte migration into wounded skin is not known, 2 theories exist - either the melanocytes migrate with the keratinocytes into the wound or, the melanocytes repopulate the wound once it has been re-epithelialised by the keratinocytes. Contrasting evidence has been shown regarding the migration of the melanocytes (Chadwick *et al.*, 2012, Breathnach, 1960). Heath *et al.* (2009) established that the migrating epithelial tongue did not contain any melanocytes. But, the melanocytes were, in fact, migrating into the wounded area. They were observed to be lagging behind the leading edge by 2-3 mm. However, in our system, we observed that the melanocytes remained in front of the keratinocytes in the wounded area at the 8 and 24 h time points. It must be borne in mind though, that our experiments were performed in an *in vitro* culture environment, and this could account for the different observations.

## CHAPTER 4: CONCLUSION

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Annually, 3.2 % of the population is burned in South Africa. This percentage is proposed to increase in time to come due to factors such as poverty, increased establishment of informal shacks and the usage of open, unattended fires. The most commonly burned areas on the body are the face, hands and feet. Clinical treatment options including autologous cellular grafting are available. However, because the priority to surgeons is mainly concerned with the closure of the wound, keratinocytes are the predominant cell type grafted onto the burned areas. The absence of melanocytes therefore leads to an insufficient amount of melanin in these grafted regions. Thus, when the wound is completely healed, it remains hypopigmented. This results in a number of psychosocial consequences, such as depression, stigmatism, discrimination and low self-esteem. The presence of melanin in the skin is crucial, not only for aesthetics, but because it protects the skin cells and their nuclei from UVR damage.

The overall aim of this study was to use 3 different ratios of melanocyte to keratinocyte co-cultures to analyse their ability to promote melanin synthesis. This was performed in order to suggest whether these contributed to improved wound healing and pigmentary outcome in burn wounds. We have shown in terms of pigmentary phenotype, surprisingly, all 3 ratios produced similar quantities of tyrosinase protein expression and activity. This suggested that the same amount melanin was produced in the ratios, therefore the keratinocyte cell number did not affect melanogenesis. In addition, no significant differences were observed in the morphology of the melanocytes. The



melanocytes in the co-cultures showed an average of 4 dendrites per cell and each dendrite was approximately 65  $\mu\text{m}$  in length. As the morphology of the cells did not change in the different ratios, it was in fact not unexpected that with the same tyrosinase expression and activity, we speculate that melanin content was the same. There was no difference in the rate of wound closure between the ratios; however, melanocyte monocultures required more than 48 h to close the wound completely. This information is an important contribution to the clinical field where the optimal outcome includes an evenly pigmented wound. The involvement of melanocytes with regard to the quantification of proliferating melanocytes in the periphery of the wound showed that there were no differences between the ratios and the time points that were investigated. This suggested that keratinocytes did not produce inhibitory factors that affected the growth of melanocytes. Furthermore, melanocytes not only migrated into the wounded area, but proliferated to increase the melanocyte population in the re-epithelialised skin. This work also showed that even though melanocytes exhibited enlarged, flattened cell morphology, this does not necessarily equate to senescence. Finally, it must be made aware that future work using the melanin assay to quantify melanin, may not be sensitive enough to detect the minute quantity of melanin in co-cultures and suggests that it is cell number dependent. For future consideration, alternative methods for melanin detection could include the use of high performance liquid chromatography or the Fontana-Masson stain.

## 4.1 Future directions

This study provides the foundation for further experiments in the field of pigmentation research and its translation to the clinical applications of wound healing medicine. Such studies could include determining which factors are secreted during wound healing to stimulate the proliferation, migration and melanin production of the melanocytes. It could be of importance to increase these specific factors to enhance the involvement of melanocytes to improve repigmentation in the wounded area. Furthermore, it would be of interest to investigate the involvement of melanocytes between the 8 and 48 h time period as in our scratch assay, most of the cellular activity occurred between these time points.

The data obtained through this study are of relevance as it can be translated to the clinical environment. Overall, the knowledge generated can be used to improve the efficacy of autologous cellular grafting in the treatment of partial-thickness burn wounds. Since no differences were observed in the 3 ratios in terms of pigmentation and wound healing, we can suggest that a ratio of 20 keratinocytes to 1 melanocyte be used in the treatment of partial-thickness burn wounds. This is an advantage as it implies that fewer melanocytes would be required to repigment the wounded area. Therefore, a smaller donor area is needed to obtain a sufficient quantity of melanocytes in the treatment, thus reducing the traumatic experience for these burn patients. However, this research is ongoing and investigations such as these are important to continue to contribute towards improved clinical treatments, therapy and outcomes.

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# APPENDIX

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Quantity sufficient (qs) is defined as enough liquid (e.g. ddH<sub>2</sub>O) that is used to make up a certain (final) volume of the solution given in liters (L) or milliliters (ml)

## A.1 10000 U/ml Penicillin/10000 µg/ml Streptomycin (100 x, 1 L)

|  |      |
|--|------|
| Penicillin (Sigma-Aldrich, USA)                                  | 6 g  |
| Streptomycin (Sigma-Aldrich, USA)                                | 10 g |
| Autoclaved double distilled water (ddH <sub>2</sub> O)           | qs   |
| Sterilise through a 0.2 µm filter and store in aliquots at -20°C |      |
| Use at 1 x dilution, after thawing                               |      |

## A.2 Hanks Buffered Salt Solution (HBSS, 1 L)

|  |        |
|--|--------|
| HBSS powder (Highveld Biological, RSA) | 9.5 g  |
| NaHCO <sub>3</sub> (Merck, RSA)        | 0.35 g |
| ddH <sub>2</sub> O                     | qs     |

## A.3 Phosphate Buffered Saline (PBS, 1 L)

|   |        |
|---|--------|
| NaCl (Merck, RSA)                                       | 8 g    |
| Na <sub>2</sub> HPO <sub>4</sub> anhydrous (Merck, RSA) | 1.26 g |
| KCl (Merck, RSA)  | 0.2 g  |
| KH <sub>2</sub> PO <sub>4</sub> (Merck, Germany)        | 0.2 g  |
| ddH <sub>2</sub> O                                      | qs     |

Adjust to pH 7.2-7.4 (or to pH 6 for SA-βgal assay) and autoclave before use



#### **A.4 0.25% Trypsin/ 0.05% Ethylenediaminetetraacetic acid (EDTA, 100 ml)**

|   |        |
|---|--------|
| Trypsin (BD Biosciences, USA)   | 0.25 g |
| EDTA anhydrous (Sigma-Aldrich, USA)   | 0.05 g |
| PBS (Appendix A.3)  | qs     |
| Adjust to pH 7.5 and sterilise through 0.2 µm filter and store in aliquots at -20°C |        |

#### **A.5 Dulbecco's Modified Eagle's Medium (DMEM, 2L)**

|   |         |
|---|---------|
| DMEM powder (Highveld Biological, RSA)                    | 27.06 g |
| NaHCO <sub>3</sub>  | 7.4 g   |
| Autoclaved ddH <sub>2</sub> O                             | qs      |
| Adjust to pH 7.2-7.4                                      |         |
| Filter sterilise through a 0.2 µm filter and store at 4°C |         |

Additives: 10% heat-inactivated fetal calf serum (FCS, Highveld Biological, RSA)  
100 U/ml penicillin/ 100 µg/ml streptomycin (1 x, Appendix A.1)

#### **A.6 4% Paraformaldehyde (PFA, 100ml)**

|   |     |
|---|-----|
| Paraformaldehyde (Merck, Germany)                       | 4 g |
| PBS   | qs  |
| Heat at 50°C to dissolve and store in aliquots at -20°C |     |

## A.7 $\beta$ gal Stain

|   |         |
|---|---------|
| Potassium ferrocyanide $K_3Fe(CN)_6$ (Sigma-Aldrich, USA)             | 5 mM    |
| Potassium ferricyanide $K_4Fe(CN)_6 \cdot 3H_2O$ (Sigma-Aldrich, USA) | 5 mM    |
| $MgCl_2$ (Merck, RSA)   | 2 mM    |
| X-gal (PEQLAB Biotechnologie, Germany)                                | 1 mg/ml |
| PBS (1 x) pH 6  | qs      |

## A.8 Mowiol

|   |       |
|---|-------|
| Mowiol (Sigma-Aldrich, USA)                 | 2.4 g |
| Glycerol (Merck, RSA)                       | 6 g   |
| Tris buffer (0.2 M) pH 8.5 (Appendix A.9)   | 12 ml |
| ddH <sub>2</sub> O                          | 6 ml  |
| Stir for a few hours at 50°C                |       |
| Centrifuge at 12000 rpm for 20 min          |       |
| Store supernatant in 1 ml aliquots at -20°C |       |

## A.9 0.2 M Tris pH 8.5 (100 ml)

|                       |        |
|-----------------------|--------|
| Tris (Merck, Germany) | 2.42 g |
| ddH <sub>2</sub> O    | qs     |
| Adjust to pH 8.5      |        |

## A.10 Complete Radio-immunoprecipitation Assay (RIPA) Buffer

|  |              |
|--|--------------|
| Complete Proteinase Inhibitor cocktail (cPIC, Appendix A.11) | 1 x          |
| Aprotinin (Roche Diagnostics, Germany)                       | 1 $\mu$ g/ml |
| PMSF (Sigma-Aldrich, USA)                                    | 0.5 mM       |
| Peptstatin A (Roche Diagnostics, Germany)                    | 1 $\mu$ g/ml |
| Incomplete RIPA buffer (Appendix A.12)                       | qs           |

### **A.11 cPIC (25 x)**

|  |          |
|--|----------|
| cPIC tablet (Roche Diagnostics, Germany) | 1 tablet |
| ddH <sub>2</sub> O                       | 2 ml     |
| Aliquot and store at -20°C               |          |
| Use at 1x                                |          |

### **A.12 Incomplete RIPA Buffer**

|   |        |
|---|--------|
| NaCl (Appendix A.13)                              | 150 mM |
| Triton X-100 (Appendix A.14)                      | 1%     |
| Sodium dodecyl sulfate (SDS, Appendix A.15)       | 0.1%   |
| Tris (pH 7.5)                                     | 20 mM  |
| Deoxycholic acid (Sigma-Aldrich, USA)             | 1%     |
| ddH <sub>2</sub> O                                | qs     |
| Sterilize through a 0.2µm filter and store at 4°C |        |

### **A.13 0.5 M NaCl (100 ml)**

|   |        |
|---|--------|
| NaCl (Merck, RSA)   | 1.46 g |
| ddH <sub>2</sub> O  | qs     |
| Dilute to necessary concentration with ddH <sub>2</sub> O |        |

### **A.14 20% Triton X-100 (1 ml)**

|  |        |
|--|--------|
| Triton X-100 (Sigma-Aldrich, USA)  | 200 µl |
| ddH <sub>2</sub> O   | 800 µl |
| Dilute to necessary concentration with ddH <sub>2</sub> O (for incomplete RIPA buffer) or 0.1 M sodium phosphate buffer (for tyrosinase enzyme activity) |        |

### **A.15 10% SDS (40 ml)**

|   |     |
|---|-----|
| SDS (Sigma-Aldrich, USA)                                  | 4 g |
| ddH <sub>2</sub> O  | qs  |
| Dilute to necessary concentration with ddH <sub>2</sub> O |     |

### **A.16 Loading dye (5 x, 20 ml)**

|  |       |
|--|-------|
| Tris (1 M) pH 6.8 (Appendix A.17)        | 6 ml  |
| Glycerol (Merck, RSA)                    | 10 ml |
| 2-β mercaptoethanol (Sigma-Aldrich, USA) | 4 ml  |
| Bromophenol blue (Sigma-Aldrich, USA)    | pinch |
| SDS                                      | 2 g   |
| Aliquot and freeze at -20°C              |       |
| Use at 1 x concentration                 |       |

### **A.17 1 M Tris pH 6.8 (100 ml)**

|                    |         |
|--------------------|---------|
| Tris               | 12.11 g |
| ddH <sub>2</sub> O | qs      |
| Adjust pH to 6.8   |         |

### **A.18 10% SDS-PAGE (20 ml)**

|  |        |
|--|--------|
| Acrylamide (30%; Sigma-Aldrich, USA)                         | 6.6 ml |
| Tris (1.5 M) pH 8.8 (Appendix A.19)                          | 5 ml   |
| ddH <sub>2</sub> O   | 8.2 ml |
| SDS (10%, Appendix A.15)                                     | 200 µl |
| APS (10%, Appendix A.20)                                     | 100 µl |
| N, N, N', N'-Tetramethylethylenediamine (Sigma-Aldrich, USA) | 10 µl  |

### **A.19 1.5 M Tris-HCl pH 8.8 (100 ml)**

|                    |         |
|--------------------|---------|
| Tris               | 18.17 g |
| ddH <sub>2</sub> O | qs      |
| Adjust to pH 8.8   |         |

### **A.20 10% Ammonium persulfate (APS)**

|                    |        |
|--------------------|--------|
| APS (Promega, USA) | 100 mg |
| ddH <sub>2</sub> O | 1 ml   |
| Store at -20°C     |        |

### **A.21 4% stacking gel (10 ml)**

|  |        |
|--|--------|
| Acrylamide (30%; Sigma-Aldrich, USA)                         | 1.3 ml |
| Tris-HCl (1M) pH6.8 (Appendix A.17)                          | 2.5 ml |
| ddH <sub>2</sub> O   | 6.1 ml |
| SDS (10%)  | 100 µl |
| APS (10%)  | 50 µl  |
| N, N, N', N'-Tetramethylethylenediamine (Sigma-Aldrich, USA) | 5 µl   |

### **A.22 Running buffer (2 L)**

|                      |        |
|----------------------|--------|
| Glycine (Merck, RSA) | 28.8 g |
| Tris                 | 6 g    |
| SDS                  | 2 g    |
| ddH <sub>2</sub> O   | qs     |

### **A.23 1 x Transfer buffer (1 L)**

|                                       |        |
|---------------------------------------|--------|
| Transfer buffer (10 x, Appendix A.24) | 100 ml |
| Methanol (Merck, RSA)                 | 200 ml |
| ddH <sub>2</sub> O                    | 700 ml |

### **A.24 10 x Transfer buffer (1 L)**

|                    |       |
|--------------------|-------|
| Glycine            | 112 g |
| Tris               | 24 g  |
| ddH <sub>2</sub> O | qs    |

### **A.25 Tris-buffered saline-Tween 20 (TBS-T, 1 L)**

|                               |        |
|-------------------------------|--------|
| Tris                          | 6.05 g |
| NaCl                          | 8.76 g |
| ddH <sub>2</sub> O            | qs     |
| Adjust to pH 7.5              |        |
| Tween 20 (Sigma-Aldrich, USA) | 1 ml   |

### **A.26 5% milk in TBS-T (10 ml)**

|   |       |
|---|-------|
| Fat-free instant milk powder (Elite, Clover, RSA) | 0.5 g |
| TBS-T   | qs    |

### **A.27 0.05% Ethylenediaminetetraacetic acid (EDTA, 100 ml)**

|   |        |
|---|--------|
| EDTA anhydrous (Sigma-Aldrich, USA)   | 0.05 g |
| PBS   | qs     |
| Adjust to pH 8 and sterilise through 0.2 µm filter and store in aliquots at -20°C |        |

### **A.28 0.05 % Trypsin/ 0.02 % EDTA (TE, 100 ml)**

|  |        |
|--|--------|
| Trypsin (BD Biosciences, USA)                                    | 0.05 g |
| EDTA anhydrous   | 0.02 g |
| ddH <sub>2</sub> O   | qs     |
| Sterilise through a 0.2 µm filter and store in aliquots at -20°C |        |

### **A.29 0.1 M Sodium Phosphate Buffer (100 ml)**

|  |       |
|--|-------|
| Sodium phosphate buffer (1 M, Appendix A.30) | 10 ml |
| ddH <sub>2</sub> O                           | 90 ml |
| Adjust pH to 7.2                             |       |

### **A.30 1 M Sodium Phosphate Buffer (200 ml)**

|   |        |
|---|--------|
| NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O (BDH Chemicals, UK) | 15.6 g |
| Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O (Merck, Germany)   | 35.8 g |
| ddH <sub>2</sub> O  | qs     |
| Adjust pH to 7-7.4  |        |

### **A.31 0.25 mM DOPA (10 ml)**

|                                 |          |
|---------------------------------|----------|
| L-DOPA (Sigma-Aldrich, USA)     | 0.0005 g |
| Sodium phosphate buffer (0.1 M) | 10 ml    |

### **A.32 0.1 N HCl (1 L)**

|                       |        |
|-----------------------|--------|
| HCl (37%; Merck, RSA) | 9.9 ml |
| ddH <sub>2</sub> O    | qs     |

### **A.33 1 M NaOH (40 ml)**

|                    |       |
|--------------------|-------|
| NaOH (Merck, RSA)  | 1.6 g |
| ddH <sub>2</sub> O | qs    |

### **A.34 0.1% Triton X-100 (20 ml)**

|              |       |
|--------------|-------|
| Triton X-100 | 20 µl |
| PBS          | 20 ml |

### **A.35 10% Bovine Serum Albumin (BSA) in PBS (20 ml)**

|   |       |
|---|-------|
| Albumin, from bovine serum (Sigma-Aldrich, USA) | 2 g   |
| PBS   | 20 ml |

### **A.36 Mowiol with anti-fade**

|   |                        |
|---|------------------------|
| Mowiol  | 1 ml                   |
| n-Propyl gallate (Sigma-Aldrich, USA)                 | tip of a small spatula |
| Vortex and heat at 50°C for 1 h, mixing occasionally  |                        |
| Centrifuge at 13000 rpm for 15-20 min                 |                        |
| Transfer supernatant to clean tube and store at -20°C |                        |